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Presence of Hepatitis E Virus (HEV) and Markers for HEV Infection in Production Swine, Human Patients with Unexplained Hepatitis, and Veterinarians in Finland

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Presence of hepatitis E virus (HEV) and markers for HEV infection in production swine, human patients with unexplained hepatitis, and veterinarians in Finland

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ACADEMIC DISSERTATION

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Abstract

Hepatitis E virus (HEV) is a non-enveloped RNA virus with a single-stranded, positive-sense genome, currently classified in the family *Hepeviridae*, and within in, proposedly in the genus *Orthohepevirus*. HEV infections are common in both humans and animals. In humans, HEV genotypes 1 and 2 (HEV-1 and HEV-2) are endemic to Asia, Africa, and Central America, where they cause large, usually waterborne, hepatitis epidemics, whereas zoonotic genotypes 3 and 4 (HEV-3 and HEV-4) cause sporadic cases worldwide. HEV-3 and HEV-4 also infect animals, and especially HEV-3 is common in swine globally. The porcine infection is usually asymptomatic. In humans, HEV-3 and HEV-4 infections are often asymptomatic or only cause mild symptoms of hepatitis, but they can also cause chronic hepatitis that can lead to liver fibrosis and cirrhosis and even death in immunocompromised patients. In this work, the presence of HEV and antibodies against HEV were confirmed in both humans and domestic pigs in Finland.

Antibodies against HEV were present in 27.6% of human patients diagnosed with acute non-A–C hepatitis and as a marker for acute hepatitis E infection, anti-HEV IgM antibodies in 11.3% of the patients. All HEV isolates obtained from the patients belonged to HEV-1. Most of the patients with acute infections had recently visited HEV-1 endemic areas in Asia, Africa, or Mexico, indicating that their infections were obtained during travels. However, the possibility of infections acquired in Finland could not be excluded, since no traveling data were available for several HEV-positive patients.

Of all production pigs of different ages investigated, from sucker-aged pigs to sows, 20.7% were positive for HEV RNA and 86.3% for antibodies against HEV. In total, samples positive for HEV RNA or anti-HEV antibodies were detected from 56.8% of separate swine farms investigated. Longitudinal follow-up studies on pigs at farrowing and fattening stages revealed that the pigs were infected with HEV at the age of 2–3 months, when the prevalence of HEV RNA-positive pigs was at its peak, 34.6%. Thereafter, the prevalence of HEV RNA-positive pigs declined to 21.1% at 3–4 months of age and to 2.9% in finisher pigs aged 5 months or older. High anti-HEV antibody seroprevalences of over 80% were detected in all age groups tested, from weaner-aged pigs to sows, indicating that hepatitis E infections are very common in production pigs in Finland. All HEVs from pigs were of HEV-3, subtype e. Genetically separate clusters of HEV isolates were obtained from different swine farms, suggesting that genetic variations in viruses from different locations occur. In addition, two different isolates were obtained from the same farm. In the follow-up studies, pig-to-pig transmission of HEV was observed. The pigs were commonly shedding HEV at the time they were transferred from farrowing farms to fattening farms, creating a possible risk of zoonotic infection for pig handlers. When pigs from HEV-negative and HEV-positive farms arrive at the same fattening farm, infection at a later age during the fattening stage must also be considered possible, which constitutes a risk for HEV entering the food chain in pork at the time of slaughter.

Antibodies against HEV are common in Finnish veterinarians, with an apparent seroprevalence of 10.2%. In statistical analysis of different background and risk factors related to veterinary work, HEV seropositivity was unexpectedly associated with working as a small animal practitioner and

negatively associated with having contacts with swine. However, contradictory to swine contacts, the seroprevalence appeared to be higher in those who had had needle stick by a needle that had previously been injected into a pig than in those who had not, suggesting that contact with blood or tissue fluid from swine might be a risk factor for HEV infection in veterinarians. In addition, those small animal practitioners who had traveled outside Europe during the previous five years appeared to be more often seropositive than those who had not, suggesting that some infections might have been travel-related. Although pigs seem to play a role in the hepatitis E infections of veterinarians, there are possibly multiple factors involved, including also other reservoirs of HEV than pigs.

HEV must be considered a possible cause of acute hepatitis in humans in Finland, especially in patients who have returned from areas endemic to HEV-1 and HEV-2. Although no human cases of possibly zoonotic HEV-3 infections acquired in Finland were detected in this study, their possibility should not be overlooked since HEV is widespread in production pigs in Finland and routes for zoonotic infection exist.

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This thesis is dedicated to the memory of my Mum.

Helsinki, March 2017 Tuija Kantala

Contents

List of original publications

This thesis is based on the following publications, referred to in the text by their Roman numerals:

I Kantala, T., Maunula, L., von Bonsdorff, C. H., Peltomaa, J., Lappalainen, M. 2009. Hepatitis E virus in patients with unexplained hepatitis in Finland. Journal of Clinical Virology, 45: 109–113.

II Kantala, T., Heinonen, M., Oristo, S., von Bonsdorff, C. H., Maunula, L. 2015. Hepatitis E virus in young pigs in Finland and characterization of the isolated partial genomic sequences of genotype 3 HEV. Foodborne and Pathogenic Diseases, 12: 253–260.

III Kantala, T., Oristo, S., Heinonen, M., von Bonsdorff, C. H., Maunula, L. 2013. A longitudinal study revealing hepatitis E virus infection and transmission at a swine test station. Research in Veterinary Science, 95: 1255–1261.

IV Kantala, T., Kinnunen, P. M., Oristo, S., Jokelainen, P., Vapalahti, O., Maunula, L. 2016. Hepatitis E virus antibodies in Finnish veterinarians. Zoonoses and Public Health, doi: 10.1111/zph.12312.

 In addition, some previously unpublished material is presented.

The author´s contribution to the publications included in this thesis was as follows:

I Participated in designing the study and was the main performer of the laboratory work. Had main responsibility for interpretation of results and writing of paper.

II Had main responsibility for designing the study and was the main performer of the laboratory work. Had main responsibility for interpretation of results and writing of paper.

III Had main responsibility for designing the study. Participated in collecting the samples and was the main performer of the laboratory work. Had main responsibility for interpretation of results and writing of paper.

IV Participated in designing the study and collecting the samples. Was the main performer of the laboratory work and the statistical analysis. Had main responsibility for interpretation of results and writing of paper.

Abbreviations

Ab	Antibody/antibodies
BLASTN	Basic Local Alignment Search Tool
bp	base pair
C-1	*Orthohepevirus* C genotype 1
C-2	*Orthohepevirus* C genotype 2
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CSC	IT Center for Science
C_t	threshold cycle
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dNTP	deoxynucleoside triphosphate
ELISA	enzyme-linked immunosorbent assay
ENANB	enterically transmitted non-A, non-B (hepatitis)
HAV	hepatitis A virus
HBV	hepatitis B virus
HCV	hepatitis C virus
HDV	hepatitis D virus
HEV	hepatitis E virus
HEV-1–HEV-7	hepatitis E virus genotypes 1–7
HVR	hypervariable region
ID	identification
IgG	immunoglobulin G
IgM	immunoglobulin M
m	missing sample
Met	methyltransferase
$MgCl_2$	magnesium chloride
N	number
NA	not assigned
Nc	not collected
NCBI	National Center for Biotechnology Information
ND	not determined
nt	nucleotide
OD	optical density
ORF	open reading frame
p	calculated probability
PBS	phosphate-buffered saline
PCP	papain-like cysteine protease
PCR	polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	ribonucleic acid
RT	reverse transcription

RT-PCR	reverse transcription polymerase chain reaction
T_m	melting temperature
TN	tris-sodium chloride phosphate-buffered saline
VLP	virus-like particle
WHO	World Health Organization

Introduction

Hepatitis E virus (HEV) infections occur in both humans and animals worldwide. The human disease, hepatitis E, manifests as two distinct diseases with different epidemiological and clinical features (Teshale et al., 2010b). HEV genotypes 1 and 2 (HEV-1 and HEV-2) only infect humans and are endemic to Asia, Africa, and Central America, where they cause enterically transmitted, usually waterborne epidemics of hepatitis E, and the disease is referred to as the endemic disease (Balayan et al., 1983; Aggarwal, 2010; Teshale et al., 2010b). HEV genotypes 3 and 4 (HEV-3 and HEV-4) are zoonotic and occur worldwide (Meng et al., 1997; Schlauder et al., 1998; Okamoto et al., 2007). In addition to humans, they also infect several different animal species (Smith et al., 2014). In humans, they cause sporadic cases of hepatitis E, referred to as the sporadic or zoonotic disease (Teshale et al., 2010b).

The existence of HEV was first suspected in 1978 during a large waterborne hepatitis epidemic in India (Khuroo, 1980). The disease was transmitted enterically, it spread from person to person, occurred in adults, and had a high attack rate and mortality among pregnant women (Khuroo, 1983). The causative agent was discovered in 1983 by Balayan and colleagues (1983) by immune electron microscopy. The full-length genome of HEV, which is a non-enveloped, single-stranded RNA virus with a positive sense genome, was sequenced in 1991 (Tam et al., 1991).

In addition to occurring in humans, the presence of HEV in animals was demonstrated in pigs in 1995 (Clayson et al., 1995a), and the first animal strain of HEV was isolated from pigs in 1997 (Meng et al., 1997). Since then, especially HEV-3 has been isolated from several species of mammals, including wild boars (Sonoda et al., 2004), different species of deer (Tei et al., 2003; Reuter et al., 2009; Forgách et al., 2010), rabbits (Zhao et al., 2009) and rats (Johne et al., 2010a). HEV-4 has been principally isolated in different animal species in China (Zhang et al., 2008b). In addition, new genotypes of HEV and HEV-like viruses with unknown potential for zoonosis have been found in several other animal species, including ferrets (Raj et al., 2012), moose (Lin et al., 2014), camels (Woo et al., 2014), and fish (Batts et al., 2011). HEV and HEV-like viruses are currently classified in the family *Hepeviridae* (Emerson et al., 2004), and within it, HEV isolates from humans, other mammalian species, and chicken are proposedly classified in the genus *Orthohepevirus*, and isolates from fish in the genus *Piscihepevirus* (Smith et al., 2014).

In swine, HEV-4 and especially HEV-3 are enzootic worldwide (Okamoto et al., 2007). Since the discovery of swine HEV, it has been shown to be common in production pigs. In the USA, 100% of adult pigs in some swine herds had antibodies against HEV (Meng et al., 1997), and HEV RNA-positive pigs have been detected in up to 100% of farms investigated in Italy and the UK (Di Bartolo et al., 2008; McCreary et al., 2008). Different HEV seroprevalences have been reported in slaughter-aged pigs in Europe, ranging from 31% (Rose et al., 2011) to 88% (Jori et al., 2016), with variations, including HEV-seronegative farms, observed between the farms investigated (Walachowski et al., 2013).

The main route of HEV transmission in pigs is fecal-oral, and the porcine infection is usually asymptomatic (Meng et al., 1997). Acute infections with fecal shedding of HEV are most common

in pigs aged 2–4 months (Cooper et al., 2005). In the feces and livers of slaughter-aged pigs, HEV RNA has been detected with rates ranging from 0% (Kaba et al., 2009; Kanai et al., 2010; Casas et al., 2011a) to 41.2% (Leblanc et al., 2007) and from 4% (Rose et al., 2011) to 75% (Walachowski et al., 2013), respectively, showing potential sources of foodborne infections via pork and pork-derived products, and of zoonotic infections for persons in contact with slaughtered pigs.

Zoonotic hepatitis E infections of foodborne origin have been linked to consumption of pig, wild boar, and deer meats and entrails, mostly uncooked or undercooked products (Matsuda et al., 2003; Li et al., 2005b; Mizuo et al., 2005; Matsubayashi et al., 2008; Renou et al., 2014), but also cooked pork and wild boar meat (Tei et al., 2003; Riveiro-Barciela et al., 2015). Persons in contact with swine, such as swine farm workers, swine veterinarians, and slaughterhouse workers, have been reported to have antibodies against HEV more often than control persons with no exposure to pigs (Drobeniuc et al., 2001; Meng et al., 2002; Bouwknegt et al., 2007; Christensen et al., 2008; Galiana et al., 2008; Krumbholz et al., 2012, 2014; Chaussade et al., 2013; Lange et al., 2016; Teixeira et al., 2016). In addition, HEV-3 has been isolated from mussels (Crossan et al., 2012; Mesquita et al., 2016), and consumption of shellfish and contact with raw sea food have been associated with acute HEV infections (Said et al., 2009; Cui et al., 2015).

The aim of this doctoral thesis was to investigate the occurrence of HEV in human patients with acute, unexplained non-A–C hepatitis, in production swine, and in veterinarians in Finland, where no previous investigations of HEV had been conducted. Specific aims were to investigate transmission of HEV and dynamics of the infection among production swine during their production cycle, to evaluate the work-related risk factors associated with HEV exposure in Finnish veterinarians, and to examine the possibility of zoonotic swine-to-human HEV infections in Finland.

2 Review of the literature

2.1 Discovery and history of hepatitis E virus (HEV)

Hepatitis E virus was discovered in 1983 by Balayan and colleagues (1983) by immune electron microscopy. Before that, in 1978, suspicions about the existence of a new hepatitis virus were raised by Khuroo (1980) during a large waterborne epidemic of jaundice with over 600 000 affected people in Kashmir, India. At this point, two causative agents for infectious viral hepatitis were already known: hepatitis A virus (HAV) and hepatitis B virus (HBV), responsible for two separate clinical diseases (Aggarwal, 2011). The possible new form of infectious hepatitis, referred to as non-A, non-B hepatitis, was characterized by a high attack rate and mortality among pregnant women, and the studies of Khuroo (1980) showed that only one of the tested serum samples from patients showed antibodies against HBV, and detected antibodies against HAV originated from previous exposure to or vaccination against HAV. In addition, the disease was transmitted enterically, spread from person to person, occurred in adults, and did not cause chronic liver infection (Khuroo et al., 1983). A few months later, Wong and colleagues (1980) reported results of their serological testing of samples collected during a large outbreak of hepatitis in New Delhi, India in 1955–1956, in addition to two smaller outbreaks in India in 1975–1976 and 1978–1979. The disease had similar characteristics, in addition to the waterborne origin, as that described by Khuroo (1980), suggesting that they were caused by the same non-A, non-B hepatitis virus. In the following years, waterborne epidemics of similar hepatitis, which came to be known as epidemic non-A, non-B hepatitis or enterically transmitted non-A, non-B (ENANB) hepatitis, were also reported from Nepal, Algeria, Burma, Pakistan, and Mexico (Kane et al., 1984; Belabbes et al., 1985; Hla et al., 1985; Leads from the MMWR, 1987; Smego and Khaliq, 1988; Velázquez et al., 1990).

Balayan and colleagues (1983) investigated an outbreak of this disease among Soviet military recruits in an army camp in Afghanistan. Balayan transmitted the disease to himself by ingesting pooled stool extracts of the patients, and following that, developed typical clinical symptoms of the acute non-A, non-B hepatitis. Approximately one month after the infection, he identified spherical virus-like particles (VLPs) of 27–34 nm in diameter in his stool samples by immune electron microscopy. However, cloning and sequencing of the virus did not succeed for several years due to the fewness of viral particles in the samples of infected patients. Later, Reyes and colleagues (1990) succeeded in partially cloning the virus, which had by that time been named hepatitis E virus (HEV), from bile obtained from an experimentally infected cynomolgus macaque. Before the successful cloning, and naming, of HEV, another non-A, non-B hepatitis virus, hepatitis C virus (HCV) was cloned (Choo et al., 1989; Kubo et al., 1989), and also the delta agent, or hepatitis D virus (HDV), had been discovered (Rizzetto et al., 1980), leading to hepatitis E virus being named with the letter ´E´. In addition, the letter ´E´ makes reference to the enteric, endemic and epidemiologic characteristics of the disease (Pérez-Gracia et al., 2016). A year later, the full-length genome of HEV was sequenced by Tam and colleagues (1991), and an enzyme immunoassay for detection of antibodies against HEV was developed by Yarbough and colleagues (1991).

Since the 1990s, intensified research of hepatitis E virus and its occurrence has led to a better understanding of the virus and the disease that it causes. In addition to occurring in humans, HEV´s presence in animals was demonstrated for the first time in 1995, when Clayson and colleagues (1995a) detected HEV RNA in pigs in Nepal. However, that RNA was not sequenced, and the first animal strain of HEV was isolated from a pig in the USA two years later by Meng and colleagues (1997). Since then, HEV and HEV-like viruses have been isolated from several species of mammals, birds, and fish (Hasqhenas et al., 2001; Tei et al., 2003; Nakamura et al., 2006; Saad et al., 2007; Zhang et al., 2008; Reuter et al., 2009; Zhao et al. 2009; Forgách et al., 2010; Johne et al., 2010a; Batts et al., 2011; Drexler et al., 2012; Raj et al., 2012; Yamamoto et al., 2012; Bodewes et al., 2013; Guan et al., 2013; Krog et al., 2013; Lin et al., 2014; Woo et al., 2014; Xu et al., 2014; Kubankova et al., 2015; Di Martino et al., 2016).

2.2 Structure and genome of HEV

HEV is a non-enveloped RNA virus (Reyes et al., 1990) with a spherical virion of approximately 27–34 nm in diameter (Balayan et al., 1983; Kane et al., 1984; Sreenivasan et al., 1984; Bradley et al., 1988). The virion has icosahedral symmetry (Xing et al., 1999) and has spikes and indentations on the surface (Bradley et al., 1987; Arankalle et al., 1988).

The genome of HEV is a positive sense, single-stranded RNA particle of approximately 7.5 kilobases in size (Tam et al., 1991), and it consists of three open reading frames (ORFs), ORF1, ORF2, and ORF3, with partial overlap (Tam et al., 1991) (Figure 1). The genome is capped at the 5' end (Kabrane-Lazizi et al., 1999), where there is also a short non-coding region of 28 nucleotides (nt). ORF1, which is the largest of the ORFs of HEV, begins at the 5' end of the genome and terminates at nt position 5109 (Panda et al., 2007). It encodes nonstructural proteins, including methyltransferase, papain-like cysteine protease, RNA helicase, and RNA-dependent RNA polymerase (Koonin et al., 1992; Emerson and Purcell, 2007). ORF2 extends from nt position 5147 to nt position 7127 (Panda et al., 2007) and encodes a glycoprotein that forms the capsid of the virus (Jameel et al., 1996). The shortest ORF, ORF3, overlaps ORF1 by one nt at its 5' end in genotypes HEV-1–HEV-3 and significantly overlaps ORF2, extending from nt position 5107 to nt position 5472. In the genome of HEV-4, ORF-3 only overlaps ORF2 (Panda et al., 2007). ORF-3 encodes a phosphoprotein that takes part in the intracellular regulation of the virus (Zafrullah et al., 1997) and promotes replication and pathogenesis (Chandra et al., 2008). At the 3' end of the genome, there is an untranslated region of 68 nt that terminates at a polyadenylated tail of approximately 150–200 nt (Tam et al., 1991).

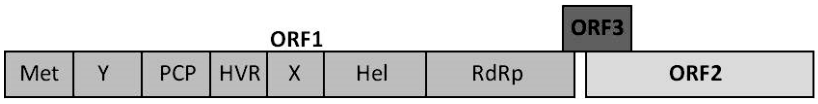

Figure 1. Genome organization of HEV-1–HEV-3 from 5 ´end to 3´ end. Met, methyltransferase; Y, Y domain; PCP, papain-like cysteine protease; HVR, hypervariable region; X, macro domain; Hel, RNA helicase; RdRp, RNA-dependent RNA polymerase.

2.3 Classification and taxonomy of HEV

After its discovery, HEV was initially suggested to group taxonomically into the *Picornaviridae* family (Miller et al., 1995), since it morphologically resembled picornaviruses (Sreenivasan et al., 1984). However, HEV turned out to be antigenically and biophysically unrelated to picornaviruses, and based on biophysical characterization of its viral particle, it was next hypothesized to be a calicivirus-like virus (Bradley et al., 1988; Tam et al., 1991). Thus, it was tentatively classified into the family *Caliciviridae*, under a separate genus *Hepevirus* (Kabrane-Lazizi et al., 1999; Panda et al., 2007). Later, however, it was observed that the order of the genes of HEV differed from that of typical caliciviruses and it was removed from the *Caliciviridae* family (Pringle et al., 1998; Green et al., 2000). Following this, HEV was classified in a separate unassigned genus 'Hepatitis E-like viruses' (Fauquet and Mayo, 2001), until it was classified as the type species of a new genus *Hepevirus* in the family *Hepeviridae* (Emerson et al., 2004). The latest proposal of taxonomy of HEV keeps it in the family *Hepeviridae*, but within it, classifies it in the genus *Orthohepevirus* and the species *Orthohepevirus* A, which includes human and swine HEV isolates, in addition to HEVs from several other mammal species (Smith et al., 2014). The genus *Orthohepevirus* also includes the species *Orthohepevirus* B, *Orthohepevirus* C, and *Orthohepevirus* D, which consist of HEVs and HEV-like viruses from both mammal species and chickens. Another genus under the family *Hepeviridae* is *Piscihepevirus*, which consists of HEV-like viruses from trout (Smith et al., 2014).

2.4 HEV genotypes

Traditionally, the HEVs infecting mammal species have been divided into four genotypes: HEV-1, HEV-2, HEV-3, and HEV-4 (Lu et al., 2006). In addition, HEV isolated from the chicken, i.e. avian HEV (Hasqhenas et al., 2001), has constituted a genotype of its own (Lu et al., 2006). During the last ten years, additional, possible new genotypes of HEV have been found from several animal species, resulting in Smith and colleagues (2014) proposing, in addition to the above-mentioned new classification of the family *Hepeviridae*, a new genotype division of HEV and HEV-like viruses presented in Table 1. This proposal designated five new HEV genotypes: genotypes HEV-5 and HEV-6 isolated from wild boars and HEV-7 isolated from camels in the species *Orthohepevirus* A, and genotypes C-1 and C-2 isolated from rats and ferrets, respectively, in the species *Orthohepevirus* C. Genotypes were not assigned for HEV isolates from chickens and bats, but they were both classified into species of their own: *Orthohepevirus* B and *Orthohepevirus* D, respectively. In addition, HEV-like viruses isolated from trouts were classified into a genus of their own, *Piscihepevirus*.

Table 1. Current, proposed classification and genotype division of HEV according to Smith et al. (2014).

Family	Genus	Species	Genotype	Most common host species
Hepeviridae	*Orthohepevirus*	*Orthohepevirus* A	HEV-1	Human
			HEV-2	Human
			HEV-3	Human, pig,
			HEV-4	Human, pig
			HEV-5	Wild boar
			HEV-6	Wild boar
			HEV-7	Camel
		Orthohepevirus B		Chicken
		Orthohepevirus C	C-1	Rat
			C-2	Ferret
		Orthohepevirus D		Bat
	Piscihepevirus	*Piscihepevirus* A		Trout

HEV-1 and HEV-2 only infect humans. HEV-1 is endemic to Africa and Asia, whereas isolates of HEV-2 have been isolated in Mexico and Africa. HEV-3 and HEV-4 are zoonotic and can infect both humans and animals. HEV-3 is distributed worldwide in humans and swine. In Europe, where it is the most frequently isolated genotype, it has been isolated from humans in several countries (Lapa et al., 2015), including Finland (Kettunen et al., 2013). In addition to swine, HEV-3 has also been frequently isolated from wild boars (Sonoda et al., 2004; de Deus et al., 2008b; Kaci et al., 2008; Martelli et al., 2008; Rutjes et al., 2010; Widén et al., 2011; Mesquita et al., 2014a; Ivanova et al., 2015) and several other animal species, most often in rabbits and different species of deer (Tei et al., 2003; Reuter et al., 2009; Zhao et al., 2009; Forgách et al., 2010). HEV-4 has most frequently been isolated in Asia, in China, Japan, Taiwan, and Vietnam in both humans and animals (Lapa et al., 2015), but also in Europe in pigs in Belgium (Hakze-van der Honing et al., 2011) and in both humans and pigs in Italy (Garbuglia et al., 2013; Monne et al., 2015). In addition to swine, HEV-4 has also been detected in wild boars (Kim et al., 2010; Sato et al., 2011) and in several other animal species in China (Zhang et al., 2008a; Xu et al., 2014; Thiry et al., 2015a).

On a nucleotide level, HEV-1 and HEV-2 isolates share a similarity of approximately 75%, as do HEV-3 and HEV-4 isolates (Lu et al., 2006). The newly identified isolates from different animal species are more distant from each other and from the HEV isolates from humans. The nucleotide similarities between the trout HEV-like virus (*Piscihepevirus*), avian HEV (*Orthohepevirus* B), rat HEV (genotype C-1), and human HEVs range between 38% and 57% (Batts et al., 2011). The HEV-like viruses from bats *(Orthohepevirus* D) share similarities of 13–52% with HEVs from other species, the lowest similarity with the trout HEV-like virus and the highest with human HEV-1 (Drexler et al., 2012). The genome of ferret HEV (genotype C-2) shared a 73% similarity with rat HEV and lower similarities of 55–61% with HEV-1–HEV-4 and avian HEV (Raj et al., 2012). The HEV-7 from camels shared nucleotide similarities of 48–76% with other known HEV

genotypes, the lowest similarity with the trout HEV-like virus and the highest with HEV-3 isolated from deer (Woo et al., 2014). Despite the genetic heterogeneity of HEV, it appears that all genotypes, or at least HEV-1–HEV-4, belong to the same serotype (Arankalle et al., 1995).

HEV-1–HEV-7 are further divided into subtypes according to Lu and colleagues (2006) and more recently according to Smith and colleagues (2016). Furthermore, Smith and colleagues (2016) proposed reference sequences for the subtypes of HEV-1–HEV-7. Based on their phylogenetic analyses, HEV-1 is divided into six (1a–1f), HEV-2 into two (2a and 2b), HEV-3 into ten (3a–3j), and HEV-4 into nine subtypes (4a–4i). The subtypes of HEV-3 have been shown to form two major clades: 3abchij and 3efg (Doceul et al., 2016; Smith et al., 2016). Lu et al. (2006) assigned nucleotide differences of HEV sequences at genotype, subtype and isolate levels and they are the most commonly used and generally accepted for differentiation of HEV sequences, and later analyses have been based on them. According to Lu et al. (2006), the differences for HEV-3 and HEV-4 are at genotype level 18.8–28.2%, at subtype level 11.4–22.8%, and at isolate level 2.0–14.8%, depending on the part of the genome sequenced. A recent study by Doceul and colleagues (2016) demonstrated that within the HEV-3 subtype clade 3abchij, the difference between the subtypes was 3.5–17.3%, and between the clade 3efg, the difference between the subtypes was 9.4–17.2% on nucleotide level.

2.5 Hepatitis E in humans

HEV is considered the most important cause of hepatitis, especially in countries with suboptimal sanitary conditions, in the areas endemic for HEV-1 and HEV-2 in Asia, Africa, and Central America. A third of the world´s population has been estimated to be exposed to HEV, and Rein and colleagues (2012) estimated that in 2005 approximately 2.1 million cases of hepatitis E occurred in the HEV-1 and HEV-2 endemic areas, causing 3.4 million symptomatic cases, 70 000 deaths and 3000 stillbirths. Since that, 2.2 million hepatitis E infections have been estimated to occur in India every year (Khuroo and Khuroo, 2016). In developed countries not endemic for HEV-1 and HEV-2, for instance USA and Europe, sporadic cases of hepatitis E were also found, and they were initially believed to be related to traveling to endemic areas (Bader et al., 1991; Skidmore et al., 1991; Zaaijer et al., 1993; Skaug et al., 1994). However, cases that were not linked to traveling were also noted (Kwo et al., 1997; Zanetti et al., 1999; Pina et al., 2000), and the possibility of locally acquired autochthonous HEV cases in countries not endemic for HEV-1 and HEV-2 was suggested. The possibility of these being of zoonotic origin was raised after the discovery of HEV in swine in 1997 (Meng et al., 1997). The strain now known as HEV-3 was found to be closely related to human HEV (Meng et al., 1997; Schlauder et al., 1998). Since then, such non-travel-related and potentially zoonotic cases of hepatitis E have been reported in several other countries throughout Europe (Widdowson et al., 2003; Amon et al., 2004; Banks et al., 2004a; Reuter et al., 2005; Colson et al., 2007a; Pérez-Gracia et al., 2007; Norder et al., 2009; Brost et al., 2010; Duque et al., 2012).

HEV usually causes an acute, self-limiting hepatitis in humans. The incubation period is typically 2–6 weeks, but it may range from nine days to up to two months. The symptoms of the disease are undistinguishable from those caused by other hepatitis viruses, especially HAV, and include fever, vomiting, anorexia, and jaundice. The duration of symptoms varies from a few weeks to more than

a month (Emerson and Purcell, 2007). Viremia starts usually two weeks after infection and lasts approximately 30 days, although HEV RNA has in some cases been detected in serum samples taken at 14 days to up to 112 days (Clayson et al., 1995b; Nanda et al., 1995; Aggarwal et al., 2000). HEV is excreted in the feces starting approximately three weeks after the infection, and the shedding lasts about 30 days, although prolonged excretion of 121 days has also been documented (Aggarwal et al., 2000; Takahashi et al., 2007). Humoral response to the infection starts with the elevation of anti-HEV immunoglobulin M (IgM) antibodies, which are usually detectable for 2–3 months after onset of symptoms (Worm et al., 2002), although in some cases they have been detected for as long as 6–7 months (Favorov et al., 1992; Bendall et al., 2008). Anti-HEV immunoglobulin G (IgG) antibodies become detectable soon after IgM and usually remain detectable for several years, up to at least 23 years (Arankalle et al., 1995; Mitsui et al., 2005; Hogema et al., 2014; Schemmerer et al., 2016), although anti-HEV IgG concentration have been seen to decrease after five years (Schemmerer et al., 2016).

Based on the research of locally acquired HEV cases in developed countries, it is apparent that the epidemiology and clinical features of hepatitis E in HEV-1 and HEV-2 non-endemic areas differ from those in endemic areas and that hepatitis E presents as two distinct diseases (Teshale et al., 2010b). The disease caused by HEV-1 and HEV-2 is referred to as the endemic disease, whereas the disease caused by HEV-3 and HEV-4 is referred to as the sporadic or zoonotic disease.

2.5.1 Endemic disease caused by HEV-1 and HEV-2

In the HEV-1 and HEV-2 endemic areas, hepatitis E in humans occurs mainly as outbreaks and epidemics that are usually caused by fecally contaminated water sources (Balayan et al., 1983; Aggarwal, 2010; Kim et al., 2014). Large epidemics often appear after monsoon or rainy seasons and after natural disasters, such as heavy rainfall and floods, which can lead to drinking water being contaminated with feces and sewage (Drabick et al., 1997; Ippagunta et al., 2007), and in crowded refugee camps with limited access to safe water (Ahmed et al., 2013; Browne et al., 2015). Outbreaks are most often caused by HEV-1, and they can affect thousands or even hundreds of thousands of people (Teshale et al., 2010b). Typically, 1–15% of a population of a community is affected in an HEV outbreak, although higher rates have also been reported, such as 25% in an outbreak in Uganda in 2007–2009 (Teshale et al., 2010a). Outbreaks caused by HEV-2 have been reported in Asia and Africa (Vélazquez et al., 1990; Maila et al., 2004). In endemic areas, the disease occurs most often in young adults, with males attacked more often than females (Aggarwal, 2011). HEV seroprevalence studies in the endemic areas have also shown that the seroprevalence is low in children, and then increases from the age of 15 years up to the peak at the age of 30 years (Rein et al., 2012). All infections are not clinical, with approximately 20–30% of those infected developing a symptomatic disease (Rein et al., 2012). In general, mortality during the epidemics is 0.2–4%, but a significantly higher attack rate of the disease occurs in pregnant women, who also have significantly higher mortality of 10–25% caused by fulminant hepatic failure, especially during the third trimester (Kumar et al., 2004). The exact reason for the predilection of the disease in pregnant women and the worse prognosis in this group still remain unknown. In addition to the fecal-oral route of infection, person-to-person transmission (Teshale et al., 2010a), vertical transmission from mother to fetus (Khuroo et al., 1995), and blood transfusion-related transmission (Khuroo et al., 2004) have also been demonstrated. Routes other

than fecal-oral are, however, considered to have a small overall significance in the outbreaks. For HEV-1, occurrence of zoonotic or foodborne infections has not been demonstrated. Human HEV-1 and HEV-2 have been shown to be able to infect non-human primates, but not other animal species (Teshale et al., 2010b). In a study of Saad and colleagues (2007) HEVs isolated from horses in Egypt clustered together with human isolates of HEV-1, but no other study has confirmed presence of HEV-1 in animals.

2.5.2 Sporadic disease caused by HEV-3 and HEV-4

The disease caused by HEV-3 and HEV-4 in humans is typically acute and self-limiting, but the majority of the infections, approximately 67–98%, are asymptomatic. The symptoms of the disease are often milder than those caused by HEV-1 and HEV-2 and can present as flu-like symptoms without jaundice (Vollmer et al., 2016a). In contrast to HEV-1 and HEV-2, HEV-3 and HEV-4 do not cause epidemics (Teshale et al., 2010b). In addition to sporadic cases of hepatitis E, they can occasionally appear as small clusters of cases, which usually have food as a point source (Matsubayashi et al., 2008; Colson et al., 2010; Guillois et al., 2015). Their main source of infection is zoonotic, with pigs as their primary reservoir (Chang et al., 2009). The sporadic disease has not been demonstrated to be more prevalent in pregnant women, and mortality among pregnant women is no higher than in the general population (Vollmer et al., 2016a).

In view of clinical cases being rare, surprisingly high HEV seroprevalences have been detected in healthy populations in HEV-1 and HEV-2 non-endemic areas. Studies on HEV seroprevalences among blood donors in Europe have revealed a wide range of seroprevalences from 1.3% to 52.5% (Boutrouille et al., 2007; Christensen et al., 2008; Mansuy et al., 2008, 2011, 2015, 2016; Masia et al., 2009; Kaufmann et al., 2011; Scotto et al., 2012; Vollmer et al., 2012; Juhl et al., 2013; Slot et al., 2013; Fischer et al., 2015; Holm et al., 2015; Lucarelli et al., 2016; O´Riordan et al., 2016; Ricco et al., 2016). Several studies have been conducted in France, where seroprevalences ranging from 3.2% (Boutrouille et al., 2007) to over 50% have been reported, and variation in the prevalences between different areas were observed (Mansuy et al., 2015). In Denmark, the seroprevalence declined with time, from 32.9% in samples collected in 1983 (Christensen et al., 2008) to 10.7% in 2015 (Holm et al., 2015). In contrast to the seroprevalences in HEV-1 and HEV-2 endemic areas, increasing age has been associated with higher seropositivity rate in developed countries, with the peak usually occurring between 40 and 70 years of age (Lewis et al., 2008; Mansuy et al., 2009, 2015, 2016; Petrović et al., 2014; Holm et al., 2015), and men more often seropositive and having the clinical disease than women (Buti et al., 2004; Lewis et al., 2008; Mansuy et al., 2009; Said et al., 2009; Meader et al., 2010; Verhoef et al., 2012). A feature distinct to HEV-3 is that the disease can progress to chronic hepatitis, which has been reported in immunocompromised patients, especially solid-organ transplant recipients. The condition can lead to liver fibrosis and cirrhosis and ultimately to death (Colson et al., 2011). Transfusion-related infections have also been documented for HEV-3 (Boxall et al., 2006; Colson et al., 2007a). Acute infections based on detected HEV RNA in donated serum samples have been reported among blood donors. Although the rates are low (< 1%), they imply potential risk of transmission to blood recipients (Vollmer et al., 2012; Hewitt et al., 2014; Lucarelli et al., 2016; O´Riordan et al., 2016). Thus, in addition to providing information about silent HEV infections in healthy persons, these studies on blood donors raise concern about the safety of blood products, especially for

immunocompromised recipients. Patients with pre-existing liver disease also have a poorer prognosis, with high mortality after HEV infection with all four genotypes (HEV-1–HEV-4) (Dalton et al., 2007; Kumar Acharia et al., 2007).

In addition to developed countries, HEV-3 especially is common in swine worldwide (Cooper et al., 2005). Reports of persons having contact with pigs, such as swine farmers and slaughterhouse workers, possessing antibodies against HEV more often than those not having contact with pigs have also been made in the HEV-1 and HEV-2 endemic areas (Chang et al., 2009; Vivek and Kang, 2011; Lee et al., 2013; Jia et al., 2014; Traoré et al., 2015), suggesting that some portion of those infections are caused by HEV-3 and HEV-4.

2.6 HEV in domestic swine

Since the isolation of the swine HEV strain in 1997 (Meng et al., 1997), studies on its occurrence in swine populations have shown that HEV-4 and especially HEV-3, previously often also referred to as the swine HEV, are enzootic in domestic pigs worldwide (Okamoto et al., 2007). HEV-4 was first detected in swine in Taiwan (Hsieh et al., 1999), and it has been found to be enzootic in swine in Japan (Takahashi et al., 2003) and in China, where it is the principal genotype in swine (Zheng et al., 2006; Chang et al., 2009; Liu et al., 2012). In Europe, HEV-4 was isolated for the first time from pigs in Belgium (Hakze-van der Honing et al., 2011).

Meng and colleagues (1997) discovered that HEV is very common in pigs, as in some swine herds in the USA 100% of adult pigs had antibodies against HEV. In studies conducted in Europe, HEV RNA has been detected in the feces of pigs in up to 100% of farms investigated. In Italy, 0–48.4% (Costanzo et al., 2015), 31% (Caruso et al., 2016), and 100% (Di Bartolo et al., 2008) of the farms investigated had HEV RNA-positive pigs. Positive pigs have been detected at 38.1% of the farms investigated in Spain (Fernández-Barredo et al., 2006), 33.3% in Slovenia (Steyer et al., 2011), 55% in both the Netherlands (Rutjes et al., 2007) and Denmark (Breum et al., 2010), 63.6% in the Czech Republic (Vasickova et al., 2009), 88–94% in Norway (Lange et al., 2016), and 100% in the UK (McCreary et al., 2008).

Domestic pigs are usually infected with HEV after maternal antibodies, originating from colostrum, disappear at the age of 1–2 months (Meng et al., 1997; Kasorndorkbua et al., 2003; de Deus et al., 2008a; dos Santos et al., 2009; Kanai et al., 2010; Feng et al., 2011). In the literature, 45–77% of breeding sows have been demonstrated to have IgG antibodies against HEV (de Deus et al., 2008a; Casas et al., 2011a). During the acute phase of the infection HEV is excreted in feces, starting from 1–2 weeks after infection and usually lasting for 3–4 weeks, but sometimes for up to 7–11 weeks (Meng et al., 1998, Halbur et al., 2001; Kasorndorkbua et al., 2004; Kanai et al., 2010), although a shorter duration of excretion lasting only 11–17 days has also been reported (Bouwknegt et al., 2008b). Thus, acute infections with fecal shedding of HEV are most common in pigs aged 2–4 months (Cooper et al., 2005; Fernández-Barredo et al., 2006; Leblanc et al., 2007; Di Bartolo et al., 2008, 2011; McCreary et al., 2008; Breum et al., 2010; Feng et al., 2011; Berto et al., 2012a; Caruso et al., 2016). Viremia starts approximately 2 weeks after fecal excretion of the virus and lasts approximately 10 days (Meng et al., 1998; Kasorndorkbua et al., 2004; Nakai et al., 2006; Bouwknegt et al., 2009). Anti-HEV IgM antibodies are usually detected at 2 weeks and anti-

HEV IgG antibodies at 2–3 weeks after the start of fecal shedding of HEV (Bouwknegt et al., 2009; Casas et al., 2009). IgM antibodies stay for longer than 10 weeks, whereas IgG antibodies usually last for the lifetime of the pigs (Meng et al., 1997; de Deus et al., 2008a).

The course of HEV antibody status and the status of markers for HEV infection starting from the birth of piglets have been described in the literature. In a study of Feng and colleagues (2011), serum samples from piglets aged 0–150 days were examined for the presence of HEV antibodies, HEV antigen, and HEV RNA. The first peak with 100% prevalence of anti-HEV IgG antibodies, without the presence of infectious markers HEV antigen and IgM antibodies, was detected at day 15, representing the peak of maternal antibodies from colostrum (Meng et al., 1997; Feng et al., 2011). In a study by dos Santos and colleagues (2009), all serum samples collected from newborn piglets before colostrum intake were negative for HEV antibodies, whereas 92.3% of the piglets were seropositive after 24 hours of colostrum intake, a finding supported by Kanai and colleagues (2010). Feng and colleagues (2011) reported that the anti-HEV antibody prevalence decreased to 59.4% at day 60 after birth, without presence of HEV antigen, showing the disappearance of the maternal antibodies. The seroprevalence then increased again from 68.8% at day 75 to 100% at day 120, falling slightly to 96.9% at day 150. In the second peak of the seroprevalence, both IgG and IgM antibodies were detected, and in addition, HEV RNA was detected at days 60–120, and HEV antigen at days 75–150, showing that the pigs were infected at the age of 2–5 months. In a comparable study by Casas and colleagues (2011a), piglets developed IgM response to infection after the maternal antibodies declined, starting from 7 weeks of age. IgG antibodies were detected starting from 13 weeks of age.

By the time of slaughter, production pigs are often cleared from the acute infection, but HEV RNA has been detected in the feces of slaughter-aged pigs, with rates ranging from 0% (Kaba et al., 2009; Kanai et al., 2010; Casas et al., 2011a) to 7.0–41.2% (Fernández-Barredo et al., 2006; Nakai et al., 2006; Leblanc et al., 2007, 2010; Di Martino et al., 2010; Di Bartolo et al., 2011; Berto et al., 2012a, 2012c; Gardinali et al., 2012) and in livers with rates ranging from 3% to 75% (Leblanc et al., 2010; Rose et al., 2011; Berto et al., 2012b; Walachowski et al., 2013; Jori et al., 2016). It has been suggested that the duration of the acute infection is sometimes longer than commonly thought or that pigs become chronically infected in an environment loaded with the virus, possibly due to incomplete immunity (Fernández- Barredo et al., 2006). In addition, re-infections, suggested to be due to short-lasting immunity, have also been considered possible in pigs (Di Bartolo et al., 2008).

Pigs usually appear clinically healthy and asymptomatic during the infection (Meng et al., 1997; Banks et al., 2004b; Fernández-Barredo et al., 2006; Zheng et al., 2006; Di Bartolo et al., 2008). On a microscopic level, mild to moderate hepatitis lesions may be apparent in the liver, including slight to moderate mononuclear inflammatory infiltrates, mainly in the periportal areas, and slight multifocal hepatocellular necrosis (Meng et al., 1997; de Deus et al., 2006). The main mode of transmission of HEV in pigs is the fecal-oral route. Bouwknegt and colleagues (2008b) estimated that one infected pig is able to infect more than eight new pigs during the acute phase. HEV RNA has also been detected in the feces of breeding sows with rates of 16.3–21.9% (Fernández-Barredo et al., 2006; 2007; de Deus et al., 2008a), suggesting that sows might also be a source of infection for suckling piglets (Fernández-Barredo et al., 2007; Di Bartolo et al., 2008; Casas et al., 2011a). Despite the presence of IgG antibodies, physiological changes during pregnancy or stress caused by farrowing have been speculated to induce reactivation of HEV replication in sows (Fernández-

Barredo et al., 2007; Casas et al., 2011a). A possible additional transmission route was suggested when HEV RNA was detected in urine of pigs (Banks et al., 2004b; Bouwknegt et al., 2009). It was proposed that urine might actually spread the virus more efficiently within and between pig pens than feces and the infection could occur orally or via droplet aspiration to the respiratory tract (Bouwknegt et al., 2009). Attempts to infect pigs via nasal secretions have also been made, with contradictory results; Andraud and colleagues (2013) demonstrated transmission between pig pens via nasal secretions, although the event was rare, whereas Kasorndorkbua and colleagues (2004) did not observe transmission via nasal secretions. In addition to pig-to-pig transmission, a contaminated environment, including feed and humans, might also be a transmission route of HEV in pigs (Di Bartolo et al., 2008). Water samples collected directly from water troughs in 16 pens were investigated in the study by Fernández-Barredo and colleagues (2006), and only one sample was positive for HEV RNA. The authors suggested that the virus might have reached the water trough through a pig´s snout that had previously been in contact with fecal material on the floor of the pen. Manure ditches from the same farms were also investigated in the study, with 50% testing positive for HEV RNA.

2.7 HEV in wild boars

In addition to domestic pigs, HEV has also been frequently isolated from wild boars. For the first time, it was detected in wild boars in Japan, and the genotype was the same as most frequently seen in production pigs, HEV-3 (Sonoda et al., 2004; Takahashi et al., 2004; Tamada et al., 2004; Li et al., 2005b; Nishizawa et al., 2005; Michitaka et al., 2007; Yano, 2007). Since then, isolation of HEV RNA from wild boars has shown that these animals are important hosts of HEV also in several European countries: Spain (de Deus et al., 2008b; Martelli et al., 2008), Germany (Kaci et al., 2008; Adlhoch et al., 2009; Vina-Rodriguez et al., 2015), where HEV RNA was also detected in wild boar serum samples collected already in 1995–1996 (Kaci et al., 2008), the Netherlands (Rutjes et al., 2010), Sweden (Widén et al., 2011; Roth et al., 2016), Italy (Caruso et al., 2015a; Kukielka et al., 2015; Martinelli et al., 2015; Mazzei et al., 2015; Montagnaro et al., 2015; Oliveira-Filho et al., 2015; Serracca et al., 2015; Di Profio et al., 2016), Portugal (Mesquita et al., 2014a), Estonia (Ivanova et al., 2015), and Slovenia (Zele et al., 2016). Furthermore, antibodies against HEV, but not HEV RNA, have been detected in wild boars in Switzerland (Burri et al., 2014). In addition to HEV-3, also HEV-4 has been detected in wild boars in South Korea (Kim et al., 2014) and Japan (Sato et al., 2011), along with the newly proposed genotypes HEV-5 and HEV-6 (Sato et al., 2011; Takahashi et al., 2011, 2014; Smith et al., 2014).

While acute HEV infections are most frequent in young domestic pigs aged 2–4 months, HEV RNA has also been detected in adult wild boars. Acute HEV infections are, however, more frequent in young wild boars. In their study in Spain, de Deus and colleagues (2008b) detected HEV RNA-positive animals more frequently among juvenile and sub-adult wild boars, 26.3% and 22.2%, respectively, than among adult animals, for whom the prevalence was 12.7%. Martelli and colleagues (2008) reported higher prevalences of HEV RNA-positive animals in Italy, the prevalence was highest, 34.8%, among wild boars of less than 12 months of age, whereas it was 25.0% among those aged 24 months or older. In addition, de Deus and colleagues (2008b) observed that HEV infection was more frequent (22.6%) in intensively reared wild boars than in those reared in open systems (19.6%) or fenced systems (17.9%). Martelli and colleagues (2008)

suggested that the difference in age-related HEV RNA occurrence between domestic pigs and wild boars might be due to HEV infections becoming chronic in wild boars or continuous re-infection as a result of short-lasting immunity.

2.8 Other confirmed or possible animal hosts of HEV

The animal species other than swine, wild boar, and chicken, from which HEV RNA has been detected are presented in Table 2. The HEV of chicken, the avian HEV, was discovered in 2001 by Hasqhenas and colleagues (2001). The genome of avian HEV and the viruses of mammals are approximately 50% similar, and avian HEV does not infect mammals (Hasqhenas et al., 2001). The most important zoonotic genotype, HEV-3, has been detected in different species of deer, rabbit, rat, mongoose, horse, monkey, and goat. HEV-4 has been detected in several animal species, including deer, cattle, and sheep, in China. New genotypes of HEV and HEV-like viruses, with unknown zoonotic potential, have been isolated from rat, trout, bat, ferret, camel, and Asian musk shrew (Table 2).

Even though HEV RNA has been detected in several animal species, there are also species from which only antibodies against HEV have thus far been detected. These species include dogs (Arankalle et al., 2001; Vitral et al., 2005; Zhang et al., 2008b; Liu et al., 2009; Geng et al., 2011a; Liang et al., 2014; McElroy et al., 2015; Wang et al., 2016), cats (Kuno et al., 2003; Okamoto et al., 2004; Mochizuki et al., 2006; Peralta et al., 2009; Liang et al., 2014), buffaloes (Shukla et al., 2007), and some deer species (Tomiyama et al., 2009). Genetic evidence of the sources of HEV antibodies in these species, with possible new animal strains of HEV, will probably be discovered in future research, adding new species into the expanding range of hosts of HEV and HEV-like viruses.

Table 2. Animal species other than swine, wild boar, and chicken, in which HEV RNA has been detected.

Species	Genotype	Country	Reference
Japanese deer (sika deer)	HEV-3	Japan	Tei et al., 2003
	HEV-4	China	Zhang et al., 2008a
Mongoose	HEV-3	Japan	Nakamura et al., 2006
Horse	HEV-1	Egypt	Saad et al., 2007
	HEV-3	China	Zhang et al., 2008b
Reeves' muntjac	ND	China	Zhang et al., 2008a
Asiatic black bear	HEV-4	China	Zhang et al., 2008a
Clouded leopard	HEV-4	China	Zhang et al., 2008a
Tufted deer	HEV-4	China	Zhang et al., 2008a
Crowned crane	HEV-4	China	Zhang et al., 2008a
Silver pheasant	HEV-4	China	Zhang et al., 2008a
Rabbit	HEV-3[1]	China	Zhao et al., 2009; Geng et al., 2011b; 2011c, 2013; Wang et al., 2013; Han et al., 2014; Xia et al., 2015
	HEV-3[1]	United States	Cossaboom et al., 2011
	HEV-3[1]	France	Izopet et al., 2012
	HEV-3[1]	Mongolia	Jirintai et al., 2012
	HEV-3[1]	Italy	Caruso et al., 2015b
	HEV-3[1]	Germany	Eiden et al., 2016[2]
	HEV-3[1]	The Netherlands	Burt et al., 2016
Roe deer	HEV-3	Hungary	Reuter et al., 2009; Forgách et al., 2010
	ND	Czech Republic	Kubankova et al., 2015
Red deer	HEV-3	Hungary	Forgách et al., 2010
	HEV-3	Spain	Boadella et al., 2010; Kukielka et al., 2015
	ND	The Netherlands	Rutjes et al., 2010
	HEV-3	Italy	Di Bartolo et al., 2015b
	ND	Czech Republic	Kubankova et al., 2015
	HEV-3	Belgium	Thiry et al., 2015b
Cattle	HEV-4	China	Thiry et al., 2015a[3]; Huang et al., 2016

Table 2. *(Continued)*

Species	Genotype	Country	Reference
Sheep	HEV-4	China	Thiry et al., 2015a[3]; Wu et al., 2015
Rat	C-1[4]	Germany	Johne et al., 2010a, 2010b, 2012
	HEV-3, C-1[4]	United States	Purcell et al., 2011; Lack et al., 2012; Debing et al., 2014
	HEV-3	Japan	Kanai et al., 2012
	C-1[4]	Vietnam	Li et al., 2013a
	C-1[4]	China	Li et al., 2013b
	C-1[4]	Indonesia	Mulyanto et al., 2013, 2014
	C-1[4]	Denmark	Wolf et al., 2013
	ND	France	Ayral et al., 2015
Cutthroat trout	NA	United States	Batts et al., 2011
Bat	NA	Africa, Central America, Europe	Drexler et al., 2012
Ferret	C-2[4]	The Netherlands	Raj et al., 2012; Smits et al., 2013
	C-2[4]	Japan	Li et al., 2015
Monkey	HEV-3	Japan	Yamamoto et al., 2012
Asian musk shrew	C-1[4]	China	Guan et al., 2013
Mink	NA	Denmark	Krog et al., 2013
Fox	NA	The Netherlands	Bodewes et al., 2013
Moose	NA	Sweden	Lin et al., 2014, 2015; Roth et al., 2016
Yak	HEV-4	China	Xu et al., 2014
Dromedary camel	HEV-7[4]	United Arab Emirates	Woo et al., 2014
Mouflon	ND	Czech Republic	Kubankova et al., 2015
Goat	HEV-3	Italy	Di Martino et al., 2016

ND, not determined; NA, not assigned.
[1]Rabbit HEV currently proposed to be a separate clade within HEV-3 (Vina-Rodriguez et al., 2015).
[2]HEV RNA detected in rabbit samples collected in 1989.
[3]HEV RNA first detected in cattle and sheep in 2010.
[4]According to the current proposed classification of the family *Hepeviridae* by Smith et al. (2014).

2.9 Hepatitis E as a zoonosis

2.9.1 Hepatitis E infections and anti-HEV antibodies in persons who have contact with animals

Possible, though not confirmed, zoonotic HEV infections from contact with animals have been reported in Japan in an owner of a pet cat that was positive for anti-HEV IgG antibodies (Kuno et al., 2003), in France in an owner of a pet pig from which HEV RNA was detected with a divergence of 8% to the RNA isolated from the owner (Renou et al., 2007), and in Spain in a slaughterhouse worker who had no risk factor for HEV infection, including traveling abroad, consuming raw meat or seafood, or receiving blood transfusions, other than his occupation (Pérez-Gracia et al., 2007). In France, acute HEV infection was reported in a medical student who practiced surgical procedures on 3-month-old pigs (Colson et al., 2007b).

Several studies conducted in Europe have investigated HEV seroprevalence in persons who have contact with animals compared with control groups (Table 3). Significantly higher seroprevalences than in control groups have been detected in persons with exposure to swine in Spain (Galiana et al., 2008) and Germany (Krumbholz et al., 2012, 2014), farmers in Denmark (Christensen et al., 2008), swine farmers in Moldova (Drobeniuc et al., 2001), pig farm workers in France (Chaussade et al., 2013), swine veterinarians in the Netherlands (Bouwknegt et al., 2007) and France (Chaussade et al., 2013), and slaughterers in Germany (Krumbholz et al., 2012). In addition, HEV seropositivity was associated with pig farming in the study of Christensen and colleagues (2008) and with close contact with swine, such as cleaning barns and assisting sows at birth, in the study of Drobeniuc and colleagues (2001). In Portugal, having been working with swine for more than 16.5 years was associated with HEV seropositivity (Teixeira et al., 2016). In Norway, veterinarians who worked with swine were twice more likely to have antibodies against HEV than other veterinarians, 22% and 9% being seropositive, respectively (Lange et al., 2016). In Estonia, pig farmers were significantly more often seropositive than hunters (Ivanova et al., 2015).

In the USA, swine veterinarians were reported to be 1.51 times more likely to be HEV seropositive than blood donors, 23.1–26.4% and 16.5–18.3% being seropositive, respectively (Meng et al., 2002). In Taiwan, the risk of HEV infection of swine farmers was reported to be 3.5 times higher than that of the general population (Lee et al., 2013). In China, significantly higher seroprevalences have been found in farmers and slaughterhouse workers than in other occupations (Chang et al., 2009; Jia et al., 2014), and farmers and veterinarians have been found to be significantly more often seropositive than control subjects, the highest seroprevalence of 49.1% was detected in swine farmers, whereas the lowest seroprevelence, 26.5%, was detected in cattle farmers (Kang et al., 2016).

Slaughterhouse workers have been shown to be significantly more often HEV antibody-positive than the control group in Germany (Krumbholz et al., 2012). At slaughterhouses, HEV has been detected in swab samples taken from floors and such tools as knives and belts, creating a risk of infection (Di Bartolo et al., 2012). In addition, HEV RNA has been found on a sample taken from a metal point used to hook the carcasses at a meat processing plant and knives and slicers at points of sale (Berto et al., 2012b).

Overall, studies indicate that persons in contact with pigs have a higher risk of HEV infection than persons not in contact with pigs or animals. However, since several studies in Europe have failed to find a significant difference between risk groups and controls (Table 3), contact with pigs is clearly not the only, and possibly not even the main risk factor for zoonotic HEV infection, and more studies are required to clarify the connection between exposure to pigs and risk of HEV infection in different occupations.

Table 3. Hepatitis E virus seroprevalences in different risk-groups with contact with animals relative to control groups in Europe.

Country	Risk group	Sero-prevalence (%) in risk group	Control group	Sero-prevalence (%) in control group	Difference statistically significant	Reference
Moldova	Swine farmers	51.1	Persons without occupational contact to swine	24.7	Yes	Drobeniuc et al., 2001
Sweden	Pig farmers	13.0	Non-farming controls	9.3	No	Olsen et al., 2006
Italy	Pig breeders	3.3	General population	2.9	No	Vulcano et al., 2007
	Abattoir workers and/or laboratory workers exposed to biological swine material	2.3	Blood donors	5.0	No	Masia et al., 2009
The Netherlands	Swine veterinarians	11	General population	2	Yes	Bouwknegt et al., 2008a
	Non-swine veterinarians	6			No	
	Persons working with animals	3.1	Persons not working with animals	1.1	No	Verhoef et al., 2012
Denmark	Farmers	50.4	Blood donors	20.6 in 2003; 31.5 in 1983	Yes	Christensen et al., 2008
Spain	Individuals exposed to swine	18.8	Individuals unexposed to swine	4.1	Yes	Galiana et al., 2008
United Kingdom	Persons with a history of close contact with a range of domestic animals	2.4	ND	ND	Exposure to pigs was not associated with seropositivity	Meader et al., 2010

Table 3. *(Continued)*

Country	Risk group	Sero-prevalence (%) in risk group	Control group	Sero-prevalence (%) in control group	Difference statistically significant	Reference
Gemany	Persons occupationally exposed to pigs	28.3	Persons with no pig exposition	15.5	Yes	Krumbholz et al., 2012
	Pig farmers	26.1			No	
	Veterinarians	22.7			No	
	Slaughterers	41.7			Yes	
	Meat inspectors	21.4			No	
	Individuals with direct contact to pigs	13.2–32.8	Individuals without direct contact to pigs	7.7–21.7	Yes	Krumbholz et al., 2014
	Hunters	21	ND	ND	No	Schielke et al., 2015
France	Pig farm workers	43.8	Individuals without working contact with animals	26.1	Yes	Chaussade et al., 2013
	Swine veterinarians	19.6	Control group from the same geographical region	8.6	Yes	
Portugal	Pet veterinarians	9.9	General population	13.3	No	Mesquita et al., 2014b
	Workers occupationally exposed to swine	30.7	Anonymous volunteers	19.9	No	Teixeira et al., 2016
Estonia	Pig farm workers[1]	13.4	ND	ND		Ivanova et al., 2015
	Hunters	4.2				
Norway	Veterinarians[2]	13	Blood donors	14	No	Lange et al., 2016
	Swine farm workers	30			Yes	

ND, Not determined.
[1]Seroprevalence in pig farm workers was significantly higher than the seroprevalence in hunters.
[2]Veterinarians who worked with swine were twice as likely to be HEV seropositive than other veterinarians.

2.9.2 Foodborne hepatitis E infections and presence of HEV in food products

Confirmed cases of acute hepatitis E linked to consumption of cooked wild boar meat and undercooked deer meat in Japan (Tei et al., 2003; Li et al., 2005b), to consumption of raw figatellu sausage in France (Renou et al., 2014), and to eating cooked pork in Spain (Riveiro-Barciela et al., 2015) have been reported. In these cases, 99.7–100% similar HEV sequences were obtained from the patient and the exact source food of the infection. The figatellu sausage is made of cold-smoked pig liver and is traditionally consumed raw. In addition to the confirmed foodborne cases, cases strongly linked to, but not similarly confirmed to resulting from the eating of uncooked, undercooked, or grilled pig liver and intestines (Mizuo et al., 2005; Matsubayashi et al., 2008), grilled pork and entrails (Miyashita et al., 2012), uncooked wild boar liver (Matsuda et al., 2003), and grilled wild boar meat (Tamada et al., 2004; Masuda et al., 2005), have been documented in Japan. In the case reported by Matsuda and colleagues (2003), two men who had eaten the same wild boar meat developed severe cases of hepatitis E, and one of the men died of fulminant hepatitis. Furthermore, an acute case potentially linked to eating wild roe deer meat has been documented in South Korea (Choi et al., 2013). In France, three clusters of HEV cases were significantly associated with consumption of pig liver-based stuffing of a spit-roasted piglet (Guillois et al., 2015). Consumption of wild boar meat and offal was associated with autochthonous HEV infection in Germany in the study of Wichmann and coworkers (2008), and consumption of pork pie, ham, and sausages purchased from a supermarket were associated with indigenous HEV infection in the UK in the study by Said and coworkers (2014). In the USA, Cossaboom and colleagues (2016) found that eating undercooked meat was associated with HEV seropositivity in college students.

Presence of HEV in several different foodstuffs or feedstock of food products, especially those of swine origin have been described in the literature. In addition to pig livers collected at slaughterhouses, HEV RNA has also been detected in porcine livers retrieved from grocery stores, with prevalences of 1.3% in the UK (Banks et al., 2010), 2% in Japan (Yazaki et al., 2003), 4% in Germany (Wenzel et al., 2011) as well as in Italy, Spain, and the Czech Republic (Di Bartolo et al., 2012), 5.7–8.8% in Canada (Wilhelm et al., 2014; 2015), 6.5% in the Netherlands (Bouwknegt et al., 2007), and 11% in the USA (Feagins et al., 2007). In addition to porcine livers, HEV RNA has also been detected in 2.5% of the 285 liver samples from hunted wild boars in France (Kaba et al., 2010). Furthermore, HEV RNA has been found in 3% of porcine meat samples collected at slaughterhouses in the Czezh Republic, Italy, and Spain (Di Bartolo et al., 2012). In France, different food products containing raw pig liver were investigated for the presence of HEV, which was detected in 25–30% of figatelli sausages and fitone, quenelle and quenelle paste, and dried or fresh liver sausages and in 3% of dried salted liver sausages (Pavio et al., 2014). HEV was also detected in 22.2% of raw and 4.3% of dry liver sausages in Italy (Di Bartolo et al., 2015a) and in 20% of raw sausages and 22% of liver sausages in Germany (Szabo et al., 2015). In a study conducted on meat and meat products illegally imported to the EU by international air travelers, HEV RNA was detected in pork, *Bovidae* meat, including meat declared as beef and buffalo, sheep and antelope meat, poultry, and guinea pig meat (Rodríguez-Lázaro et al., 2015). To support the possibility of foodborne infections, Takahashi and coworkers (2012) have demonstrated that infective HEVs were present in porcine livers sold as food, and Berto and colleagues (2013a) have confirmed that infectious HEV was present in pig liver sausage.

Furthermore, Said and colleagues (2009) have reported that consumption of shellfish was associated with acute HEV infections during an outbreak on a cruise ship that returned to the UK after a world cruise. In the outbreak, 33 acute infections, 11 of which were symptomatic, and 192 seropositive cases were confirmed. In China, a higher risk of HEV infection was detected in seafood processing workers who had direct contact with raw seafood than in those who were less exposed (Cui et al., 2016). The likely source of HEV contamination in mussels and shellfish is the water, instead of the meat being infected with the virus (Crossan et al., 2012; Donia et al., 2012; Gao et al., 2015; Mesquita et al., 2016). In the studies of Crossan and coworkers (2012) and Mesquita and coworkers (2016), HEV RNA was detected in 85% of investigated mussels in the UK and in 15% of those investigated in Spain, respectively, and the isolated viruses were of genotype HEV-3 in both studies. In addition, HEV-4 was found to be excreted in milk of HEV-infected cows in China, suggesting a possible new source of infection (Huang et al., 2016).

HEV-3 has also been detected in strawberries in Canada (Brassard et al., 2012), although the HEV was suspected of originating from river water used for irrigation of the strawberry field. In the study by Maunula and coworkers (2013), an HEV-positive pack of frozen raspberries collected from point-of-sale was found, but molecular analysis of the sample was not possible. HEV has also been found in 3% of samples collected from salad vegetables supply chain in Greece, Serbia, and Poland (Kokkinos et al., 2012). The likely source of HEV contamination in berries and salad is contaminated irrigation water. HEV has been detected in irrigation water of vegetables (Kokkinos et al., 2016), as well as in surface water samples (Steyer et al., 2011; Williamson et al., 2011; Lazić et al., 2015; Marcheggiani et al., 2015; Givens et al., 2016) and wastewater samples (Masclaux et al., 2013 Beji-Hamza et al., 2015; Myrmel et al., 2015), and wastewater potentially contaminates the natural waters used for irrigation, in addition to seawater that contaminates shellfish.

The results of numerous studies on several different food products demonstrate that pig- and wild boar-derived sources of foodborne HEV infections are common, and the meat of other animal species, in addition to seafood, can also be sources of infection.

2.10 Diagnosis of hepatitis E

When HEV was first detected, the diagnosis was based on immune electron microscopy (Bradley et al., 1987) and fluorescent blocking antibody assay and HEV antigen detection in hepatocytes, dependent on light microscopy (Krawczynski and Bradley, 1989). The diagnosis has since then been facilitated by the development of serological methods for anti-HEV antibody detection and molecular techniques for detection of HEV RNA. HEV does not replicate efficiently in vivo, and growing it in cell culture has proven to be extremely difficult. Thus, no efficient cell culture systems for HEV exist to facilitate HEV detection and diagnosis of hepatitis E, although cell culture models have been developed and used in replication studies (Tanaka et al., 2007; Takahashi et al., 2010; Berto et al., 2013b; Okamoto et al., 2013).

Serological diagnosis of all four human infecting HEV genotypes, HEV-1–HEV-4, with one test is possible since they all belong to the same serotype (Khudyakov and Kamili, 2011), and numerous in-house and commercial ELISA (enzyme-linked immunosorbent assay) tests are in use in both

research and patient diagnosis. However, contradictory results have been reported for commercially available ELISA tests, demonstrating their different performance characteristics, including sensitivities ranging between 17% and 100% (Mast et al., 1998; Herremans et al., 2007a; Norder et al., 2016; Perez-Gracia et al., 2016). Immunoblot assays can be used to confirm the results of ELISA tests, but no consensus exists regarding their need in accurate detection of anti-HEV antibodies. For instance, according to Herremans and colleagues (2007a), the combination of ELISA and immunoblot tests is essential for acceptable specificity and sensitivity in serological diagnosis of HEV, whereas Vollmer and coworkers (2016a) recently reported that immunoblot did not increase the informative value of the serological results obtained by ELISA. Furthermore, there is currently no gold standard for serological diagnosis of HEV, and research is needed to develop and provide more reliable diagnostic tests (Rossi-Tamisier et al., 2013; Krumbholz et al., 2014; Norder et al., 2016; Vollmer et al., 2016b).

Real-time RT-PCR (reverse transcription polymerase chain reaction) is a sensitive and specific method for detection of HEV RNA in both sera and feces. No gold standard for PCR diagnosis of HEV is available, although The World Health Organization (WHO) has established a standard for HEV RNA, which allows comparison of different PCR assays for HEV detection (Baylis et al., 2013). The in-house methods, as well as their sensitivities, used in research and diagnosis vary between tests and laboratories (Baylis et al., 2011). Furthermore, there is currently no consensus of which genomic region should be used for genotyping HEV. However, whole-genome sequencing is getting common and is preferred for classification of HEV (Liu et al., 2008). Since the viremic phase of HEV infection is relatively short, a negative PCR result should not be used to rule out HEV infection (Perez-Gracia et al., 2016). However, detecting HEV RNA, especially with simultaneous serological detection of anti-HEV IgM antibodies, is a sensitive and specific indication of an acute infection.

2.11 Prevention of hepatitis E infections

In HEV-1 and HEV-2 endemic areas, especially in developing countries, essential prevention procedures of hepatitis E infections are reducing exposure to HEV by improving sanitary conditions and providing clean drinking water, and inducing immunity by using vaccinations, which are discussed in section 2.11.1 (Kamar et al., 2012; Pérez-Gracia et al., 2015). Travelers to these areas should make sure to only use uncontaminated drinking water and to remember the basic hygienic procedures including washing hands with soap and water.

To prevent foodborne HEV infections, good hygienic practices are needed in food preparation, starting from appropriate safety measures during storage and handling of uncooked pork –bearing in mind the possibility of cross-contamination from pork to the surfaces and other foodstuff simultaneously handled – to ensuring that meat and meat products are thoroughly cooked. In the study by Barnaud and colleagues (2012), complete inactivation of HEVs in paté-like preparations was achieved when heated at 71˚C for 20 minutes. Feagins and coworkers (2008) demonstrated that when HEV-contaminated livers were boiled or stir-fried for 5 minutes, the virus was also completely inactivated, whereas inactivation was not achieved when the liver samples were incubated at 56˚C for 1 hour. It would also be important to inform people, especially risk populations including pregnant women, immunocompromised individuals, and persons with liver

conditions, of the potential risk of hepatitis E from raw or uncooked pork, wild boar, and deer meat (Pérez-Gracia et al., 2015). Recently, Guerrero-Latorre and colleagues (2016) reported that UV disinfection and flocculation-chlorination sachets reduced HEV concentration in water. These and other possible measures to reduce HEV contamination in water warrant further study.

In farm practices, preventive methods, such as wearing gloves and washing hands with soap and water, appear to reduce HEV exposure to humans (Meng, 2013; Pavio et al., 2015). In the study of Schielke and colleagues (2015), hunters who wore protective gloves when disemboweling wild boars were found to be significantly less often HEV seropositive than those who did not wear gloves. Wilhelm and colleagues (2016) found in their logistic regression analysis study that requiring shower-in from visitors to swine farms was a significant predictor of lower detection of HEV RNA. Wilhelm and colleagues (2016) also found that if farms obtained finisher pigs from several different sources, odds of HEV RNA detection from pigs increased. Further investigations are needed to identify the types of preventive and biosecurity measures that could be used to limit HEV transmission among pigs and also to clarify how HEV persists in farm surroundings to determine which cleaning and disinfection procedures can be used to eliminate it.

2.11.1 HEV vaccine

Since all four HEV genotypes infecting humans belong to the same serotype, HEV is considered a good candidate for vaccine development (Purcell and Emerson, 2008). Thus far, only one vaccine, prepared by a Chinese research group and known as HEV 239 vaccine, has been registered and available for use in China, under the name Hecolin (Li et al., 2005a; Zhang et al., 2009; Zhu et al., 2010). The vaccine is based on HEV-1, and more studies are required to determine its efficacy against HEV-3, although it has been proven to provide protection against HEV-4 infections. WHO has evaluated the use of the vaccine in preventing and controlling HEV infections in terms of safety, immunogenicity, efficacy, and cost-effectiveness, and as a result, routine use of the vaccine in HEV endemic countries was not recommended. Nevertheless, countries could use it as a part of a prevention program in special situations, such as outbreaks of HEV, and for travelers and pregnant women (WHO, 2015). For prevention of zoonotic HEV infections, using vaccines in animals has also been considered. Liu and colleagues (2014) studied the efficacy of the Chinese vaccine on rabbits exposed to HEV-4 and HEV-7, and found the vaccine to be effective. Future research is needed to investigate the efficacy of the vaccine in other animals, especially pigs, and to evaluate the possibility of reducing the risk of zoonotic infections by vaccinating pigs (Meng, 2013; Liu et al., 2014).

3 Aims of the study

The objectives of this work were to investigate the occurrence of hepatitis E virus in selected human populations and in production swine in Finland, to investigate transmission of HEV among production swine during their production cycle, and to evaluate the work-related risk factors associated with HEV exposure in Finnish veterinarians.

Specific aims were as follows:

1. To investigate the occurrence and transmission of hepatitis E infections in production pigs in Finland, and to identify genetic divergence of HEV strains occurring in production pigs at different swine farms (II, III, unpublished)

2. To investigate the occurrence of hepatitis E infections in selected human populations in Finland: in patients with unexplained acute hepatitis (I) and in veterinarians, in addition to evaluating the work-related risk factors associated with HEV exposure in veterinarians (IV)

3. To compare the seroprevalences obtained by ELISA tests detecting total anti-HEV, anti-HEV IgG, or anti-HEV IgM antibodies in human serum samples (unpublished)

4. To compare the sensitivity and suitability of two sets of primers and conventional RT-PCR methods targeting different regions of the HEV genome for sequencing and genetic characterization of swine HEV (unpublished)

5. To examine the possibility of zoonotic swine-to-human hepatitis E infections in Finland (I–IV, unpublished).

4 Materials and methods

4.1 Study designs, sampling, and data collection

4.1.1 Hepatitis E infections in human patients with unexplained acute hepatitis (I)

In Study I, to investigate whether HEV is the cause of hepatitis in Finnish human patients with unexplained acute hepatitis, 105 serum samples, collected between March 2000 and January 2008 from 97 patients who had all been diagnosed and treated for acute hepatitis, were selected from the archives of the Department of Virology, Helsinki University Hospital and tested for the presence of anti-HEV IgG and IgM antibodies. Of the patients, 53 were males and 44 females. The patients were aged 14–87 (mean 41.5) years. Sixty-two patients originated from the Helsinki and Uusimaa Hospital District area, and the samples from them had all been tested and found negative for HAV and/or HBV, and most of them also for HCV. Thirty-five patients originated from all over Finland, and their samples (N=43) had been tested and found positive for anti-HEV IgG antibodies. Samples that were found HEV antibody-positive were also tested for the presence of HEV RNA. Information on traveling history was available for some of the patients.

4.1.2 Occurrence of HEV in production swine in different production stages and presence of HEV at swine farms (II, III, unpublished)

To achieve an overview of HEV occurrence in production pigs in different production stages and the presence of HEV at swine farms in Finland, a total of 568 fecal and/or serum samples from a total of 376 individual pigs, and pooled fecal samples collected from the floors of 114 pig pens were examined for HEV RNA, and serum samples from 249 pigs were examined for anti-HEV antibodies (Table 4). The samples were collected during 1998–2011 from a total of 37 separate swine farms: farms 1–29 (in Studies II and III, and unpublished studies) and A–H (in Study III), in addition to a swine test station on three occasions in 2007, 2010 (in Study III), and 2011 (in unpublished studies), and from a slaughterhouse in 2011 (in unpublished studies). Samples collected from 34 separate swine farms, in addition to the swine test station in all three occasions, and the slaughterhouse were examined for HEV RNA. Serum samples collected from pigs at six separate farms, in addition to the swine test station in 2007 and 2010, and the slaughterhouse were examined for the presence of anti-HEV antibodies.

The sampled pigs were divided into groups according to their production stage: sucker pigs (age approximately 0–4 weeks), weaner pigs (age approximately 2–3 months), grower pigs (age approximately 3–4 months), finisher pigs (age $\geq$ 5 months), and sows (Table 4). According to the production stages of pigs sampled, the swine farms were categorized as farrowing farms or fattening farms. At farrowing farms sucker pigs and/or weaner pigs, and at fattening farms grower pigs and/or finisher pigs were sampled. Of the 37 separate swine farms, 28 farms were farrowing farms and 13 farms were fattening farms. At seven farms, pigs in both farrowing and fattening

stages were sampled. Only sows were sampled at three farms. Samples from sucker pigs were collected from six, samples from weaner pigs from 28, samples from grower pigs and finisher pigs both from nine, and samples from sows from four separate farms. In addition, samples from grower pigs were collected at the swine test station on all three occasions, and samples from finisher pigs at the swine test station in 2007 and 2011, and at the slaughterhouse. From 118 pigs, samples were collected twice or more often, up to six times, at different ages during the study.

The swine test station from which samples of pigs during the fattening stage were collected was comparable with a commercial fattening farm. The station operated as a center for testing production traits of pigs from Finnish swine herds, and litters of pigs from all over Finland were sent to the station at the age of 2–3 months, which is the age the pigs are normally sent to a fattening farm. On arrival, the litters were divided into pens in one unit of the station in mixed groups that were kept stationary during a three-month raising period, which ended at slaughter at the age of 5–6 months.

Table 4. Numbers of swine samples tested for HEV RNA and anti-HEV total antibodies according to the production stage of the pigs, together with the number of farms from which the samples were collected.

Production stage and age of pigs from which samples were collected	Numbers of types of samples tested			Number of farms from which samples were collected
	Fecal and serum samples from individual pigs tested for HEV RNA	Pooled fecal samples collected from floors of pens tested for HEV RNA	Serum samples tested for anti-HEV antibodies	
Sucker pigs (0–4 weeks)	67	23	0	6
Weaner pigs (2–3 months)	162	30	2	28
Grower pigs (3–4 months)	123	36	24	12[1]
Finisher pig (≥ 5 months)	210	25	136	12[2]
Sows	6	0	87	4
Total	568	114	249	41[3]

[1]Samples were collected from 9 separate swine farms, and from the swine test station on three occasions in 2007, 2010, and 2011.
[2]Samples were collected from 9 separate swine farms, and from the swine test station on two occasions in 2007 and 2010, and the slaughterhouse in 2011.
[3]In total, samples were collected from 37 separate swine farms of which samples from pigs in only one production stage were collected from 30 farms, and samples from pigs in two or more production stages were collected from 11 farms, and from the swine test station in three occasions in 2007, 2010, and 2011, and from the slaughterhouse in 2011.

4.1.3 Dynamics of hepatitis E infections in production swine during the farrowing and fattening stages and at the time of slaughter (II, III)

To investigate hepatitis E infections in young pigs during their farrowing stage, HEV status of pigs was monitored at follow-up studies at two farrowing farms (farms 1 and 2) in October–December 2010 in Study II. Fecal samples examined for the presence of HEV RNA were collected from 20 piglets at both farms on four occasions. At the beginning of the samplings, the piglets were aged 2–4 weeks (mean 26 days), and the samples were collected every 2–4 weeks until the age of 10–12 weeks or until the pigs were transferred to fattening farms. At farm 1, pigs from four litters and at farm 2 pigs from five litters were investigated.

To examine the occurrence and transmission of HEV among pigs during their fattening stage, two follow-up studies, in 2007 and 2010, in which the HEV status of pigs from 8–12 weeks of age until 18–22 weeks of age was monitored at the swine test station, were conducted in Study III. In 2007, 40 pigs originating from 11 farms were investigated. From them, fecal samples were collected on three occasions, during the pigs´ first, fifth, and 11th week at the station. In 2010, 36 pigs originating from eight farms were examined. From them, fecal samples were collected on six occasions, at two-week intervals between the pigs´ first and 11th week at the station. All fecal samples were examined for the presence of HEV RNA. In addition, blood samples were collected from the pigs at the slaughterhouse at the age of 20–24 weeks, from 37 pigs in 2007 and from 27 pigs in 2010, and examined for the presence of anti-HEV total antibodies.

4.1.4 Genetic divergence of HEV strains occurring in production swine at different swine farms (II, III, unpublished)

For examination of genetic divergence of HEV strains occurring in production swine at different swine farms in Finland, phylogenetic analyses of HEV sequences obtained from samples from pigs from different swine farms in Studies II and III, as well as some sequences obtained in previously unpublished studies were used. In total, 53 sequences from the ORF1 region, encoding the RNA polymerase gene, and 25 sequences from the ORF2 region, encoding the structural gene of the HEV genome, were analyzed. From 19 samples, both ORF1 and ORF2 sequences, from 34 samples only ORF1 sequence, and from six samples only ORF2 sequence were obtained and included in the analysis.

The 53 ORF1 sequences, 26 of which were unique (GenBank accession numbers KJ825678–KJ825690 (II), JN585116–JN585127 (III), and KX266169 (unpublished)) were obtained from samples from pigs from nine separate farms (1, 2, 5, and 7–12) and from the swine test station in 2007 and 2010. The 25 ORF2 sequences, 18 of which were unique (GenBank accession numbers KJ825691–KJ825702 (II) and KX266163–KX266168 (unpublished)) were obtained from samples from pigs from eight separate farms (1–4, 8–10, and 12) and from the swine test station in 2007 and 2010. From farm 1, sequences obtained from samples collected in three different years (2005, 2009, and 2010), and from farm 2, sequences obtained from samples collected in two different years (2009 and 2010) were included in the analysis.

4.1.5 Comparison of sensitivity and suitability of two sets of primers and conventional RT-PCR methods targeting different regions of the HEV genome for sequencing and genetic characterization of swine HEV (II, unpublished)

The PCR and sequencing results from Study II were used to compare two sets of primers and two different RT-PCR methods for sequencing and genetic characterization of the swine HEVs. Primer pair ISP and lmR, with conventional RT-PCR method, targeted the RNA polymerase gene in the ORF1 region of the HEV genome, and the set of four primers, 3156–3159, with conventional nested RT-PCR method, targeted the structural gene in the ORF2 region of the HEV genome (Table 5). To compare the methods with regard to their ability and sensitivity to amplify the HEV RNA-positive samples for sequencing, the proportions of sequences obtained by both primer sets according to the C_t (threshold cycle) values of the samples in real-time RT-PCR were compared. The suitability of the two amplicons for genotyping swine HEV was assessed by comparing the results of their phylogenetic analysis.

Table 5. Sets of primers and probe used for real-time RT-PCR for HEV RNA detection and for conventional RT-PCR for sequencing.

Primer/ probe	Target gene	PCR method	Used in studies	Sequence	Location	Polarity	Reference
HEV2F	Structural gene (ORF2)	Real-time RT-PCR	I– IV	5'-GTGGTTTCTGGGGTGAC-3'	5262–5278	Positive	Modified for this study from Orrù et al., 2004
HEV2R			I–IV	5'-GGGGTTGGTTGGATGAATA-3'	5311–5329	Negative	
JVHEVP			II–IV	5'-TGATTCTCAGCCCTTCGC-3´	5284 –5301	Probe	Jothikumar et al., 2006
ESP	RNA polymerase (ORF1)	Conventional nested RT-PCR	I	5'-CATGGTAAAGTGGGTCAGGGTAT-3'	4213-4235	Positive	Zhai et al., 2006
EAP			I	5'-AGGGTGCCGGGCTCGCCGGA-3'	4576-4595	Negative	
ISP			I	5'-GTATTTCGGCCTGGAGTAAGAC-3'	4232–4253	Positive	
IAP			I	5'-TCACCGGAGTGYTTCTTCCAGAA-3'	4561-4583	Negative	
ISP	RNA polymerase (ORF1)	Conventional RT-PCR	II, III	5'-GTATTTCGGCCTGGAGTAAGAC-3'	4232–4253	Positive	Zhai et al., 2006
lmR			II, III	5'-GTCCAGGCCGAYCTAACTAA-3'	4511–4530	Negative	Designed for this study.
3156	Structural gene (ORF2)	Conventional nested RT-PCR	II	5´-AAYTATGCMCAGTACCGGGTTG-3´	5687–5708	Positive	Meng et al., 1997
3157			II	5´-CCCTTATCCTGCTGAGCATTCTC-3´	6395–6417	Negative	
3158			II	5´-GTYATGYTYTGCATACATGGCT-3´	5972–5993	Positive	
3159			II	5´-AGCCGACGAAATYAATTCTGTC-3´	6298–6319	Negative	

4.1.6 Presence of anti-HEV antibodies in Finnish veterinarians and evaluation of possible work-related risk factors associated with HEV exposure in veterinarians (IV)

In Study IV, to investigate the occurrence of hepatitis E infections among Finnish veterinarians, the presence of anti-HEV total antibodies was examined from serum samples from 333 veterinarians, including veterinary students. The samples were collected during the 2009 Annual Veterinary Congress in Helsinki, Finland. In addition to veterinarians, 52 non-veterinarians who attended the congress, including veterinary nurses and product and medical sales representatives, also participated in the study. The samples were collected from voluntary participants after signing an informed consent. Samples that were positive for total HEV antibodies, as well as 93 randomly selected samples that tested negative for total HEV antibodies were further examined for the presence of anti-HEV IgM antibodies to detect possible acute infections. In addition, HEV IgM-positive samples were further tested for the presence of HEV RNA. The study was performed according to the World Medical Association Declaration of Helsinki, and it was approved by the Ethic Committee of Helsinki University Central Hospital.

To evaluate possible risk factors for hepatitis E infections related to veterinary work, an electronic questionnaire that covered relevant background information, including risk factors for zoonotic hepatitis E infection, was composed. Most of the participants of the study completed the questionnaire: information on occupation (veterinarian/non-veterinarian) and sex was given by all 385 participants (333 veterinarians and 52 non-veterinarians), and further background information by 357 participants (310 veterinarians and 47 non-veterinarians). The veterinarians were assigned to job specialty categories according to their areas of work as follows: (1) municipal veterinarians, (2) small animal practitioners, and those who worked (3) in a slaughterhouse, (4) in a horse clinic, (5) in a laboratory, (6) in an office, (7) in some other area of work, and (8) those who did not provide data on their area of work. Some veterinarians included in categories (1) and (3)–(5) worked in more than one area. Most, 89.1% (147/165), of the municipal veterinarians reported performing farm visits. The distribution of the samples according to age, sex, occupation (veterinarian/non-veterinarian), and practice specialty categories of the veterinarians is presented in Table 6. As possible risk factors for HEV infection, occupation (veterinarian/non-veterinarian), sex, age, work-related contacts with pigs and wild boars, history of traveling abroad during the preceding five years, history of traveling to Asia, Africa, or Mexico during the previous six months, having ever received hospital care abroad, and eating pork were analyzed for both veterinarians and non-veterinarians. In addition, the veterinary job specialties, as well as needle sticks by a needle that had previously been injected either into any animal or a pig were analyzed for veterinarians only. None of the participants had been diagnosed or suspected of having hepatitis during the 12 months preceding the study.

Table 6. Sampled subjects according to age, sex, occupation (veterinarian/non-veterinarian), and practice specialty categories of the veterinarians.

	Number of sampled subjects (number of male subjects, if any, is given in parentheses)										
		Veterinarians in practice specialty categories									
Age	Veterinarian	(1) Municipal veterinarian[1]	(2) Small animal practitioner only	(3) Slaughter-house[1]	(4) Horse clinic[1]	(5) Laboratory[1]	(6) Office only	(7) Other	(8) Data not provided	Non-veterinarian	Total
< 20	0	0	0	0	0	0	0	0	0	1	1
20–29	43 (1)	17 (1)	8	4	4	6 (1)	0	13	2	19 (2)	62 (3)
30–39	118 (3)	65 (2)	31 (1)	15 (1)	1	19	6	6	1	15 (1)	133 (4)
40–49	84 (13)	46 (10)	23 (3)	9 (2)	3 (1)	6	5	5	1	8 (1)	92 (14)
50–59	45 (12)	24 (8)	8 (1)	13 (1)	2 (2)	5	4 (2)	3	0	3 (1)	48 (13)
60–69	16 (9)	11 (7)	2 (1)	3 (2)	1	2 (2)	1	1	1 (1)	1	17 (9)
70–79	3 (3)	2 (2)	1 (1)	2 (2)	1 (1)	2 (2)	0	0	0	0	3 (3)
Not provided	24 (5)	0	0 (0)	0	0	0	0	0	1 (1)	5	29 (5)
Total	333 (46)	165 (30)	73 (7)	46 (8)	12 (4)	40 (5)	16 (2)	28	6 (2)	52 (5)	385 (51)

[1]All veterinarians who reported belonging to the practice specialty category, only or in addition to other practice specialty categories.

4.1.7 Comparison of seroprevalences obtained by ELISA tests detecting anti-HEV total antibodies and anti-HEV IgG antibodies in human serum samples (IV, unpublished)

For comparison of seroprevalences obtained by Axiom HEV-Ab ELISA test (Axiom GmbH, Bürstadt, Germany) detecting anti-HEV total antibodies and *recomWell* HEV IgG test (Mikrogen GmbH, Neuried, Germany) detecting anti-HEV IgG antibodies, a total of 130 human serum samples from Study IV were analyzed by both ELISA tests. The analyzed samples included samples that tested anti-HEV total antibody positive, in addition to randomly selected samples that tested negative for anti-HEV total antibodies by Axiom HEV-Ab ELISA test in Study IV, and the same samples were also tested by *recomWell* HEV IgG test.

4.1.8 Sampling and sample storage (I–IV, unpublished)

Fecal samples from individual pigs were collected directly from their rectums, and pooled fecal samples from pig pens were collected from the floors of pig pens (II, III unpublished). All fecal samples were collected using clean plastic gloves. Blood samples were collected at the slaughterhouse during bleeding (III, unpublished). All samples were transported to the laboratory within 24 hours in a cool box. After arrival, serum was separated from the blood samples by centrifugation. Serum samples of human patients with unexplained hepatitis were selected for testing from the archives of the Department of Virology and Immunology, Helsinki University Hospital, HUSLAB (I). Blood samples from veterinarians and non-veterinarians were collected from voluntary participants of the Annual Veterinary Congress at the congress site, after obtaining a signed informed consent (IV). Sera were separated from blood samples within 12 hours of sampling. All fecal and serum samples were stored at –20°C until analyzed.

4.2 Anti-HEV antibody detection (I, III, IV, unpublished)

For detection of anti-HEV antibodies, five different ELISA tests were used: Genelabs Diagnostics ELISA kits (Genelabs Diagnostics Pte., Ltd., Singapore) for detection of anti-HEV IgM and IgG in human sera (I), Axiom HEV-Ab ELISA kit (Axiom) for detection of anti-HEV total antibodies in human sera (IV) and swine sera (III, unpublished), and *recom*Well HEV IgM and IgG kits (Mikrogen) for detection of anti-HEV IgM (IV) and IgG (unpublished) antibodies, respectively, in human sera. In addition, two immunoblot assays for detection of anti-HEV IgM and IgG in human sera (Mikrogen) were used (I). All tests were performed and the cut-off values for each test calculated according to manufacturers´ instructions.

4.3 RNA extraction (I–IV, unpublished)

The same RNA extraction method was used throughout the studies. RNA was extracted from 140 µl of serum or 10% stool suspensions in TN (tris-sodium chloride) or PBS (phosphate-buffered saline) buffer using QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer´s instructions to reach a final volume of 60 µl of RNA.

4.4 HEV RNA detection by real-time RT-PCR (I–IV, unpublished)

Detection of HEV RNA from both human and swine samples was performed by real-time RT-PCR targeting a 68 base pair (bp) long fragment of the structural gene of HEV in ORF2 using primers HEV2F and HEV2R (Table 5). In Study I and partly in Study III, SYBR Green chemistry was used, and in Study II, partly in Study III and in Study IV, as well as in the unpublished swine studies, a modified method using TaqMan chemistry with probe JVHEVP (Table 5) was used. In both methods, amplification was performed with Rotor-Gene 3000 Instrument (Corbett Life Sciences, Sydney, Australia). In data analysis of both methods, the threshold of the PCR was set at 0.02. A cut-off C_t of 40 was used in all parts of the study (I–IV, partly the unpublished), except in the comparison of sensitivity and suitability of two sets of primers and conventional RT-PCR methods targeting different regions of the HEV genome for sequencing and genetic characterization of swine HEV (unpublished), where all samples with a C_t value were analyzed.

With SYBR Green chemistry, before the amplification, reverse transcription (RT) reaction was performed at 42°C for 60 minutes using a reaction solution containing 2.5 µM reverse primer HEV2R, GeneAmp 1 x PCR buffer containing 15 mM of $MgCl_2$ (Applied Biosystems, Foster City, CA, USA), 10 mM DTT (Roche Diagnostics GmbH, Mannheim, Germany), 8.8 mM dNTP mix, 20 units Recombinant RNasin Ribonuclease Inhibitor (Promega Corporation, Madison, WI, USA), 25 units Expand Reverse Transcriptase (Roche Diagnostics), and 3 µl of extracted RNA. Amplification was then carried out using 3 µl of the synthesized cDNA, 0.5 µM primers HEV2F and HEV2R, and Quantitect SYBR Green PCR Kit (Qiagen) according to the manufacturer´s instructions. The amplification was carried out under the following conditions: initial activation at 95°C for 15 minutes, followed by 50 cycles of 94°C for 15 seconds, 54°C for 45 seconds, and 72°C for 15 seconds. Melting curves were measured at 67–95°C, and products with a melting temperature (T_m) of 80–81°C were considered positive.

With TaqMan chemistry, the amplification was carried out as one-step RT-PCR using 3 µl of the synthesized cDNA, 0.9 µM primers HEV2F and HEV2R, 0.3 µM probe JHVEVP, and QuantiTect Probe RT-PCR Kit (Qiagen) according to the manufacturer´s instructions. The amplification was carried out under the following conditions: RT reaction at 50°C for 30 minutes, initial activation at 95°C for 15 minutes, followed by 50 cycles of 95°C for 15 seconds, 55°C for 45 seconds, and 72°C for 45 seconds.

4.5 Sequencing (I–III, unpublished)

HEV RNA-positive samples were further amplified for sequencing using two conventional RT-PCR methods, a nested RT-PCR (I, III, unpublished) and a one-step RT-PCR (II, III, unpublished). For the nested RT-PCR, two sets of primers were used: external primers ESP and EAP and internal primers ISP and IAP, targeting a 352-bp fragment of the RNA polymerase gene in ORF1 (I), and external primers 3156 and 3157 and internal primers 3158 and 3159, targeting a 348-bp fragment of the structural gene in ORF2 (II, unpublished) (Table 5). With both primer sets, the first-round RT-PCR was performed using Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer´s protocol, with 0.8 μM each of both external primers, 8 units Recombinant RNasin Ribonuclease Inhibitor (Promega Corporation), and 3 μl of extracted RNA. With both primer sets, the RT-PCR started with an RT reaction at 50°C for 60 minutes and initial activation at 95°C for 15 minutes. With primers ESP and EAP, the amplification then continued as follows: 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and with primers 3156 and 3157 as follows: 45 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute. A final extension at 72°C for 10 minutes was carried out with both primer sets. The first-round RT-PCR product was then amplified in the second-round PCR. With internal primers ISP and IAP, 2.5 μM each of the primers were used, and with internal primers 3158 and 3159, 0.5 μM. Otherwise, the amplification was performed in both studies with HotStarTaq Plus Master Mix Kit (Qiagen) according to the manufacturer´s instructions, and 1 μl of the product from the first-round PCR. The reaction was carried out in the following conditions: initial activation at 95°C for 5 minutes, followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

With the one-step RT-PCR, primers ISP and lmR targeting a 298-bp fragment of RNA polymerase gene in ORF1 (Table 5) were used in amplification for sequencing. The amplification was performed using Qiagen OneStep RT-PCR Kit (Qiagen) according to the manufacturer´s protocol, with 0.8 μM each of the primers, 8 units Recombinant RNasin Ribonuclease Inhibitor (Promega Corporation), and 3 μl of extracted RNA. RT-PCR was carried out in the following conditions: RT reaction at 50°C for 60 minutes, initial activation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes.

The final PCR products were separated on 2.0% or 3.5% MetaPhor agarose gel (Lonza, Basel, Switzerland) stained with ethidium bromide. If this was not successful, a band-stab PCR technique described by Bjourson and Cooper (1992) was used (II, III). Briefly, in this technique, the appropriate band in the agarose gel was stabbed with an injection needle, which was then dipped into a fresh PCR reaction mix that was prepared as in the previous PCR, without the template. Amplification was then carried out as in the previous PCR amplification, and after that, the product was separated on agarose gel. In Study III, after the one-step RT-PCR, a second-round PCR with the same primers using HotStarTaq Plus Master Mix Kit (Qiagen) was also used if needed. All PCR products that produced the expected DNA fragment were sequenced at the DNA Sequencing Service, Institute of Biotechnology, University of Helsinki using Applied Biosystems ABI3130XL Genetic Analyzer or ABI3730 DNA Analyzer.

4.6 Genotyping and phylogenetic analysis (I–III, unpublished)

All sequences obtained were compared with HEV sequences in NCBI GenBank using BLASTN program, and with each other using BioEdit program (http://www.mbio.ncsu.edu). For phylogenetic trees, additional, previously reported HEV sequences available in GenBank were used. Multiple alignment analysis and bootstrap value calculation for the nucleotide sequences were conducted using ClustalW program through the IT Center for Science (CSC) (I, III), ClustalX 2.1 or Clustal Omega programs (http://www.clustal.org) (II), or MEGA6 (unpublished). The phylogenetic trees were constructed using NJplot program (I–III) or MEGA6 (the trees presented in Figures 1 and 2 in the thesis) with a neighbor-joining method, using Kimura´s correction for multiple substitutions.

4.7 Statistical analysis (IV)

In Study IV, where the work-related risk factors associated with HEV exposure in veterinarians were evaluated, the prevalences of total HEV antibody-positive subjects were compared according to the potential risk factors for HEV infection. Two-by-two tables and test statistics were used to evaluate the differences between seroprevalences. For all tests, a 2-tailed $p < 0.05$ was considered statistically significant.

5 Results

5.1 Hepatitis E infections in human patients with unexplained acute hepatitis (I)

Anti-HEV antibodies were detected by both ELISA test and immunoblot in 22.7% (22/97) of the patients with unexplained acute hepatitis tested (Table 7). Of all serum samples tested, 27.6% (29/105) were found anti-HEV antibody-positive. Of the seropositive patients, 54.5% (12/22) were positive for only anti-HEV IgG, and 45.5% (10/22) for either anti-HEV IgM only or for both anti-HEV IgM and IgG. HEV RNA was detected in the samples of 8.2% (8/97) of the patients tested (Table 7). All HEV RNA-positive samples were from patients who were also HEV-seropositive. Seven of the eight RNA-positive patients were also anti-HEV IgM-positive, whereas one was positive for only anti-HEV IgG. The results indicated that 11 patients had acute infections, including the 10 patients who were positive for anti-HEV IgM antibodies in addition to the one positive for HEV RNA and anti-HEV IgG antibodies. Another 11 patients who were only positive for anti-HEV IgG antibodies had had an earlier HEV infection. Five HEV sequences were obtained from the eight HEV RNA-positive samples. All of the isolates belonged to genotype HEV-1, based on the nucleotide similarity of 97–99% they shared with HEV-1 isolates in GenBank.

Traveling history was available for eight of the 11 patients with acute HEV infections. Seven of them had traveled to HEV-1 endemic areas in India, Bangladesh, Thailand, Nepal, and Vietnam, whereas one had traveled to Spain. Seven of the 11 patients with acute infections were males, and four were females; both sexes were aged 22–50 years (mean 30.7 years for males and 33.8 years for females). The onsets of their hepatitis symptoms had been from less than 1 week to less than 2 months before the samplings.

Table 7. Results of anti-HEV IgM and IgG and HEV RNA detections of patients with unexplained hepatitis.

Number of patients = 97	Anti-HEV antibody positive by ELISA (% of all patients)	ELISA result confirmed by immunoblot (% of patients seropositive by ELISA)	HEV RNA positive by real-time RT-PCR (% of patients confirmed seropositive by immunoblot)
IgM and IgG positive	11[1] (11.3)	10[1] (90.9)	7 (70.0)
IgM positive only	2 (2.1)	0 (0)[2]	0 (0)
IgG positive only	23[1] (23.7)	12 (52.2)	1 (8.3)
Total positive	35[1] (36.1)	22 (62.9)	8 (36.4)

[1]For one patient who was positive for both IgM and IgG by ELISA, only the IgG positivity was confirmed by immunoblot. This patient is thus also included in the 'IgG-positive only' category, and thus, the total number of patients who were anti-HEV antibody-positive by ELISA is 35.
[2]Insufficient sera left to perform immunoblots.

5.2 Occurrence of HEV in production swine in different production stages and presence of HEV at swine farms (II, III, unpublished)

In total, HEV RNA was detected in 20.7% (78/376) of individual pigs sampled (Table 8) and in 25.4% (29/114) of pooled fecal samples collected from pig pens (Table 9). Anti-HEV total antibodies were detected in 86.3% (215/249) of the pigs investigated (Table 8). From 56.8% (21/37) of separate swine farms investigated either HEV RNA-positive or anti-HEV antibody-positive pigs or HEV RNA-positive pooled fecal samples were found.

HEV RNA-positive samples were most frequently detected among weaner pigs and grower pigs, at the age of 2–4 months. No HEV RNA-positive sucker pigs were observed. Of samples from weaner pigs 34.6% (56/162), from grower pigs 21.1% (26/123) and from finisher pigs 2.9% (6/210) were HEV RNA-positive. No HEV RNA-positive sows were seen. Anti-HEV antibodies were examined in pigs of all production stages, except sucker pigs, and seropositive pigs were detected in all examined stages. The seropositivity rate was 100% (2/2) in weaner pigs, 95.8% (23/24) in grower pigs, 87.5% (119/136) in finisher pigs, and 81.6% (71/87) in sows.

HEV RNA-positive samples were detected at 50.0% (17/34) of the separate swine farms from which samples were examined for HEV RNA, as well as at the swine test station on all three occasions (2007, 2010 and 2011), and at the slaughterhouse (Tables 8 and 9). At least one HEV RNA-positive pig was detected at 50.0% (14/28) of separate farrowing farms, and at 53.8% (7/13) of separate fattening farms. HEV RNA-positive fattening-aged pigs were also detected at the swine test station on all three occasions, and at the slaughterhouse. Anti-HEV total antibody-positive pigs were detected at each of the six separate farms from which samples were examined for antibodies, as well as at the swine test station on two occasions in 2007 and 2010, and at the slaughterhouse.

Table 8. Numbers of individual pigs testing positive and numbers of pigs tested for HEV RNA and anti-HEV total antibodies according to the farms of origin and the production stage of the pigs.

	Number of samples testing positive/number of samples tested (%)											
	Farrowing farms				Fattening farms							
	Sucker pigs (0-4 weeks)		Weaner pigs (2-3 months)		Grower pigs (3-4 months)		Finisher pigs (≥ 5 months)		Sows		Total	
Farm	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab
1	0/25 (0.0)	Nc	20/25 (80.0)	Nc	4/10 (40.0)	10/10 (100)	1/19 (5.3)	10/10 (100)	0/6 (0.0)	24/26 (92.3)	25/85 (29.4)	44/46 (95.7)
2	0/25 (0.0)	Nc	15/25 (60.0)	Nc	Nc	Nc	1/9 (11.1)	Nc	Nc	Nc	16/59 (27.1)	Nc
3	0/5 (0.0)	Nc	1/4 (25.0)	Nc	0/1 (0.0)	Nc	0/2 (0.0)	Nc	Nc	Nc	1/12 (8.3)	Nc
4	0/2 (0.0)	Nc	0/5 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/7 (0.0)	Nc
5	0/5 (0.0)	Nc	0/5 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/10 (0.0)	Nc
6	0/5 (0.0)	Nc	0/5 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/10 (0.0)	Nc
7	Nc	Nc	2/2 (100)	2/2 (100)	4/5 (80.0)	5/5 (100)	0/3 (0.0)	3/3 (100)	Nc	Nc	6/10 (60.0)	10/10 (100)
8	Nc	Nc	2/4 (50.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	2/4 (50.0)	Nc
10	Nc	Nc	5/8 (62.5)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	5/8 (62.5)	Nc
11	Nc	Nc	2/3 (66.7)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	2/3 (66.7)	Nc
12	Nc	Nc	2/3 (66.7)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	2/3 (66.7)	Nc
13	Nc	Nc	Nc	Nc	0/9 (0.0)	8/9 (88.9)	2/7 (28.6)	8/8 (100)	Nc	Nc	2/16 (12.5)	16/17 (94.1)
14	Nc	Nc	0/12 (0.0)	Nc	0/9 (0.0)	Nc	Nc	Nc	Nc	Nc	0/21 (0.0)	Nc
15	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	13/14 (92.9)	Nc	13/14 (92.9)
16	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	21/27 (77.8)	Nc	21/27 (77.8)
17	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	Nc	13/20 (65.0)	Nc	13/20 (65.0)
18	Nc	Nc	Nc	Nc	Nc	Nc	0/2 (0.0)	Nc	Nc	Nc	0/2 (0.0)	Nc

Table 8. *(Continued)*

	Number of samples testing positive/number of samples tested (%)											
	Farrowing farms				Fattening farms							
	Sucker pigs (0-4 weeks)		Weaner pigs (2-3 months)		Grower pigs (3-4 months)		Finisher pigs (≥ 5 months)		Sows		Total	
Farm	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab
19	Nc	Nc	Nc	Nc	Nc	Nc	0/5 (0.0)	Nc	Nc	Nc	0/5 (0.0)	Nc
20	Nc	Nc	Nc	Nc	Nc	Nc	0/4 (0.0)	Nc	Nc	Nc	0/4 (0.0)	Nc
21	Nc	Nc	Nc	Nc	Nc	Nc	0/35 (0.0)	Nc	Nc	Nc	0/35 (0.0)	Nc
22	Nc	Nc	0/1 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/1 (0.0)	Nc
23	Nc	Nc	2/6 (33.3)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	2/6 (33.3)	Nc
24	Nc	Nc	0/4 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/4 (0.0)	Nc
25	Nc	Nc	1/3 (33.3)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	1/3 (33.3)	Nc
26	Nc	Nc	1/2 (50.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	1/2 (50.0)	Nc
27	Nc	Nc	0/3 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/3 (0.0)	Nc
28	Nc	Nc	0/1 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/1 (0.0)	Nc
29	Nc	Nc	0/5 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/5 (0.0)	Nc
A	Nc	Nc	0/3 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/3 (0.0)	Nc
B	Nc	Nc	0/6 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/6 (0.0)	Nc
C	Nc	Nc	0/6 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/6 (0.0)	Nc
D	Nc	Nc	0/3 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/3 (0.0)	Nc
E	Nc	Nc	0/2 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/2 (0.0)	Nc
F	Nc	Nc	1/7 (14.3)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	1/7 (14.3)	Nc
G	Nc	Nc	0/6 (0.0)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	0/6 (0.0)	Nc
H	Nc	Nc	2/3 (66.7)	Nc	Nc	Nc	Nc	Nc	Nc	Nc	2/3 (66.7)	Nc

Table 8. *(Continued)*

	Number of samples testing positive/number of samples tested (%)											
	Farrowing farms				Fattening farms							
	Sucker pigs (0-4 wks)		Weaner pigs (2-3 months)		Grower pigs (3-4 months)		Finisher pigs (≥ 5 months)		Sows		Total	
Farm	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab	HEV RNA	HEV Ab
Test station 2007	Nc	Nc	Nc	Nc	2/43 (4.7)	Nc	0/38 (0.0)	32/37 (86.5)	Nc	Nc	2/81 (2.5)	32/37 (86.5)
Test station 2010	Nc	Nc	Nc	Nc	11/36 (30.6)	Nc	0/36 (0.0)	23/28 (82.1)	Nc	Nc	11/72 (15.3)	23/28 (82.1)
Test station 2011	Nc	Nc	Nc	Nc	5/10 (50.0)	Nc	Nc	Nc	Nc	Nc	5/10 (50.0)	Nc
Slaugh-ter-house 2011	Nc	Nc	Nc	Nc	Nc	Nc	2/50 (4.0)	43/50 (86.0)	Nc	Nc	2/50 (4.0)	43/50 (86.0)
Total	0/67 (0.0)	Nc	56/162 (34.6)	2/2 (100)	26/123 (21.1)	23/24 (95.8)	6/210 (2.9)	119/136 (87.5)	0/6 (0.0)	71/87 (81.6)	88/568[1] (15.5)	215/249 (86.3)

Ab, antibodies; Nc, not collected.
[1]118 pigs were tested twice or more often (up to six times) at different ages during the study. Thus, the total number of pig individuals tested for HEV RNA was 376, and the total number of HEV RNA positive pig individuals was 78.

Table 9. Numbers of pooled fecal samples collected and pooled fecal samples positive for HEV RNA according to the farms of origin and the production stage of the pigs in the pens sampled.

	Numbers of samples testing positive/numbers of samples tested (%) per production stage				
	Farrowing farms		Fattening farms		
Farm	Sucker pigs (0-4 weeks)	Weaner pigs (2-3 months)	Grower pigs (3-4 months)	Finisher pigs (≥ 5 months)	Total
1	0/4 (0.0)	3/8 (37.5)	Nc	Nc	3/12 (25.0)
2	0/3 (0.0)	0/1 (0.0)	3/4 (75.0)	Nc	3/8 (37.5)
3	0/4 (0.0)	3/6 (50.0)	1/2 (50.0)	Nc	4/12 (33.3)
4	0/4 (0.0)	4/7 (57.1)	Nc	Nc	4/11 (36.4)
5	0/4 (0.0)	0/4 (0.0)	2/4 (50.0)	Nc	2/12 (16.7)
6	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	Nc	0/12 (0.0)
9	Nc	Nc	2/2 (100)	Nc	2/2 (100)
Test station 2007	Nc	Nc	11/20 (55.0)	0/25 (0.0)	11/45 (24.4)
Total	0/23 (0.0)	10/30 (33.3)	19/36 (52.8)	0/25 (0.0)	29/114 (25.4)

5.3 Dynamics of hepatitis E infections in production swine during the farrowing and fattening stages and at the time of slaughter (II, III)

During the farrowing stage (II) all pigs examined remained negative for HEV RNA between the ages of 2 and 6 weeks. The age of onset of fecal HEV RNA excretion varied between the two farms examined; at farm 2, every second pig started excreting the virus already at the age of 7–8 weeks, whereas at farm 1, all pigs started excretion only at the age of 10–12 weeks, i.e. just before or at the time of transfer to fattening farms (Table 10). At that time, practically all pigs sampled at both farms, 96.6% (28/29), were HEV RNA-positive. On farm 2, the last sample was not available for 11 pigs that had already been sent to a fattening farm at the time of the delayed last sampling (one week delay for reasons beyond our control). In total, 87.5% (35/40) of the pigs were HEV RNA-positive during the study. Three pigs on farm 2 had two consecutive positive samples.

During the fattening period (III) in the first follow-up conducted in 2007, 35% (14/40) of the pigs were HEV RNA-positive after arriving at the swine test station from different farrowing farms at the age of 8–12 weeks. One of the pigs remained positive four weeks later, while the rest of the pigs (26/40) remained HEV RNA-negative until the end of follow up, at 5–6 months of age. No transmission of the virus between the pigs was observed in 2007. In the second follow-up conducted in 2010, three pigs were HEV RNA-positive after arriving at the station at the age of 8–12 weeks. New infections were detected after the first sampling, suggesting that transmission of the virus within pens occurred; six new pigs were positive on week 3 at the age of 10–14 weeks and five new pigs on week 5 at the age of 12–16 weeks. No positive pigs were detected beyond this point. In total, 38.9% (14/36) of the pigs were positive during the 2010 survey (Table 11). At the time of slaughter, anti-HEV total antibodies were detected in 86.5% (32/37) and 78.6% (22/28) of the pigs from which a serum sample was available in 2007 and in 2010, respectively.

Table 10. Results of HEV RNA detection of the pigs at two farrowing farms included in the follow-up study of HEV occurrence during the farrowing stage.

Farm 1						Farm 2					
		HEV RNA detection (C_t values of the positive samples)						HEV RNA detection (C_t values of the positive samples)			
Pig ID	Litter ID	Day 1, age 2–4 weeks	Day 13, age 4–6 weeks	Day 34, age 7–9 weeks	Day 55, age 10–12 weeks	Pig ID	Litter ID	Day 1, age 3–4 weeks	Day 13, age 5–6 weeks	Day 28, age 7–8 weeks	Day 55, age 11–12 weeks
1	a	–	–	–	+ (31.93)	21	e	–	–	–	+ (33.65)
2	a	–	–	–	+ (22.06)	22	e	–	–	–	m
3	a	–	–	–	+ (34.94)	23	e	–	–	–	+ (33.32)
4	a	–	–	–	+ (27.50)	24	e	–	–	+	m
5	b	–	–	–	+ (31.73)	25	f	–	–	+	+ (35.09)
6	b	–	–	–	+ (32.40)	26	f	–	–	–	–
7	b	–	–	–	+ (34.00)	27	f	–	–	+	m
8	b	–	–	–	+ (36.00)	28	f	–	–	+	m
9	b	–	–	–	+ (32.70)	29	g	–	–	–	m
10	c	–	–	–	+ (33.70)	30	g	–	–	–	+ (34.20)
11	c	–	–	–	+ (32.30)	31	g	–	–	–	m
12	c	–	–	–	+ (35.40)	32	g	–	–	+	m
13	c	–	–	–	+ (33.10)	33	h	–	–	+	+ (23.82)
14	c	–	–	–	+ (33.40)	34	h	–	–	+	m
15	d	–	–	–	+ (31.30)	35	h	–	–	+	+ (20.88)
16	d	–	–	–	+ (36.10)	36	h	–	–	+	m
17	d	–	–	–	+ (32.80)	37	i	–	–	–	m
18	d	–	–	–	+ (33.90)	38	i	–	–	–	+ (35.03)
19	d	–	–	–	+ (34.00)	39	i	–	–	+	m
20	d	–	–	–	+ (34.40)	40	i	–	–	–	+ (22.20)
Total number of positive samples/ samples tested		0	0	0	20/20 (100%)	Total number of positive samples/ samples tested		0	0	10/20 (50%)	8/9 (89%)

ID, identification; +, positive; –, negative; m, missing sample.

Table 11. Results of HEV RNA and anti-HEV total antibody detection of the pigs included in the 2010 follow-up study of HEV occurrence during the fattening stage at the swine test station.

			Results of detections				
			HEV RNA				HEV Ab
Pen	Pig ID	Farm ID	Week 1, age 2–3 months	Week 3, age 2.5–3.5 months	Week 5, age 3–4 months	Weeks 7, 9, 11, age 3.5–5.5 months	Week 14, age 5–6 months
1	1-10	B	–	+	–	–	+
	2-10	B	–	–	–	–	+
	3-10	B	–	–	+	–	+
	4-10	B	–	–	+	–	+
	5-10	B	–	–	+	–	+
	6-10	C	–	–	–	–	+
	7-10	E	–	–	–	–	+
	8-10	E	–	–	–	–	+
	9-10	G	–	+	–	–	–
2	10-10	C	–	–	–	–	m
	11-10	C	–	–	–	–	+
	12-10	C	–	–	–	–	m
	13-10	D	–	–	–	–	m
	14-10	D	–	–	–	–	–
	15-10	D	–	–	–	–	–
	16-10	F	+	–	–	–	+
	17-10	F	–	–	–	–	–
	18-10	H	+	–	–	–	m
	19-10	H	–	–	–	–	+
3	20-10	A	–	–	+	–	m
	21-10	A	–	–	–	–	–
	22-10	A	–	–	+	–	+
	23-10	B	–	+	–	–	m
	24-10	C	–	–	–	–	+
	25-10	H	–	–	–	–	m
	26-10	G	–	–	–	–	–
	27-10	G	–	–	–	–	+
	28-10	H	+	–	–	–	+
4	29-10	C	–	–	–	–	+
	30-10	H	–	–	–	–	+
	31-10	H	–	+	–	–	+
	32-10	H	–	–	–	–	+
	33-10	G	–	–	–	–	m
	34-10	G	–	+	–	–	+
	35-10	G	–	+	–	–	+
	36-10	H	–	–	–	–	+
Total number of positive samples/ samples taken			3/36 (8.3%)	6/36 (16.7%)	5/36 (13.9%)	0	22/28 (78.6%)

Ab, antibodies; ID, identification; +, positive sample; –, negative sample; m, missing sample.

5.4 Genetic divergence of HEV strains occurring in production swine at different swine farms (II, III, unpublished)

A total of 78 partial HEV sequences obtained in Studies II and III, and in unpublished studies were analyzed: 53 ORF1 sequences from nine different swine farms and the swine test station from 2007 and 2010, and 25 ORF2 sequences from eight separate farms and the test station from 2007 and 2010. According to phylogenetic analysis and comparison with HEV sequences in GenBank, all sequences obtained in this study belonged to genotype HEV-3, and within it, subtype e, as assigned by Lu et al. (2016). In the phylogenetic trees constructed, the ORF1 sequences formed eight separate clusters (Figure 2), whereas the ORF2 sequences formed seven separate clusters (Figure 3). Between the clusters, the ORF1 sequences shared nucleotide similarities of 87.50–93.89% and the ORF2 sequences 86.47–94.40%. Within the clusters, the ORF1 sequences shared nucleotide similarities of 94.91–100%, and the ORF2 sequences 95.11–100%.

Of the eight clusters in the ORF1 tree (Figure 2), five clusters only included sequences from one farm each: all sequences from farm 1 from three different years (2005, 2009, and 2010) as well as all sequences from farm 2 from two different years (2009 and 2010), in addition to all sequences from farms 5, 7, and 11, from which sequences from one year per farm were obtained, were arranged as separate clusters. In three clusters, sequences from several different farms were included. Sequences from farm 10 and the swine test station from 2007 were both divided into two different clusters, whereas sequences from farms 8, 9, and 12, and the swine test station from 2010 all belonged to one cluster each. In first of the three mixed clusters there were sequences from farms 10 and 12, in the second, sequences from farm 9 and the swine test station from 2007, and in the third, sequences from farms 8 and 10, and from the swine test station from 2007 and 2010.

Of the seven clusters in the ORF2 tree (Figure 3), four clusters only included sequences from one farm each. As in the ORF1 tree, all sequences from farm 1 from three different years and all sequences from farm 2 from two different years were arranged as separate clusters. Sequences from farms 10 and 12 also formed clusters of their own. In three mixed clusters sequences from several farms were included. The sequences from the swine test station from 2007 were divided into two different clusters, whereas sequences from farms 3, 4, 8, and 9, and the swine test station from 2010 all belonged to one cluster each. The first of the mixed clusters included sequences from farms 3 and 4. In the second, sequences from farm 9 and the swine test station from 2007 were included, and in the third, there were sequences from farm 8 and from the swine test station from 2007 and 2010.

On the amino acid level, all of the ORF2 sequences encoding the structural gene of HEV were 100% identical. The ORF1 sequences encoding the RNA polymerase gene of HEV were divided into six groups, between which variation of 1–4 amino acids at positions 1440 (serine or proline), 1447 (valine or alanine), 1450 (glutamic acid or aspartic acid), 1461 (glycine or valine), and 1462 (threonine or serine) in reference to the HEV prototype Burma strain (M73218) were observed. The amino acid sequences obtained from the swine test station in 2007 and 2010 in Study III were divided into three groups, within which the sequences shared 100% identity with each other. The first group was formed by four sequences obtained from pooled fecal samples from pens and from an individual pig from farm 11 at the test station in 2007 and it was referred to as ´the pen 2007-like isolate´. The second group, referred to as ´the pig 2007-like isolate´, included sequences that

were obtained from samples from individual pigs from farms 10 and 12 in 2007. The third group constituted all of the sequences from the swine test station from 2010 and the six sequences obtained from pooled fecal samples and samples from individual pigs from farms 8 and 10 in 2007, and it was referred to as ´the 2010-like isolate´. Amino acid sequences from farms 2, 5, 7, and 9 from Study II grouped into ´the pen 2007-like isolate´, as did one previously unpublished sequence from farm 1 from 2005. Overall, the amino acid sequences from farm 1 were divided into four separate groups, as the rest of sequences from farm 1 formed three new groups: one sequence from 2005 formed a group of its own, as did one sequence from 2010, whereas the rest of the sequences from 2009–2010 grouped together.

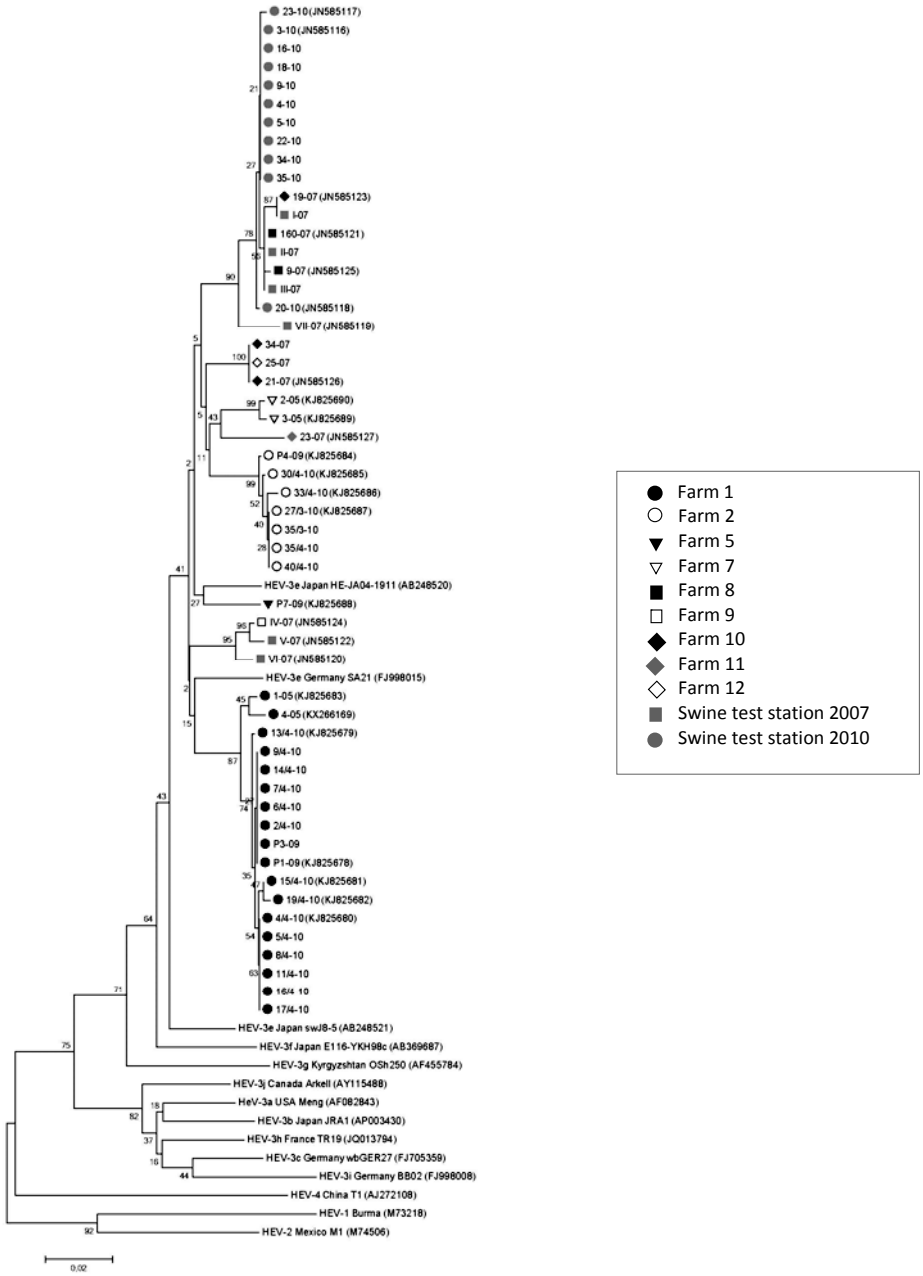

Figure 2. Phylogenetic tree based on a 216-nt fragment within the RNA polymerase gene in ORF1, showing the 53 isolates from nine farms and the swine test station in 2007 and 2010 from this study, together with 14 previously reported HEV isolates of HEV-1, HEV-2, HEV-4, and HEV-3 subtypes a–c and e–j as proposed for reference sequences by Smith et al. (2016).

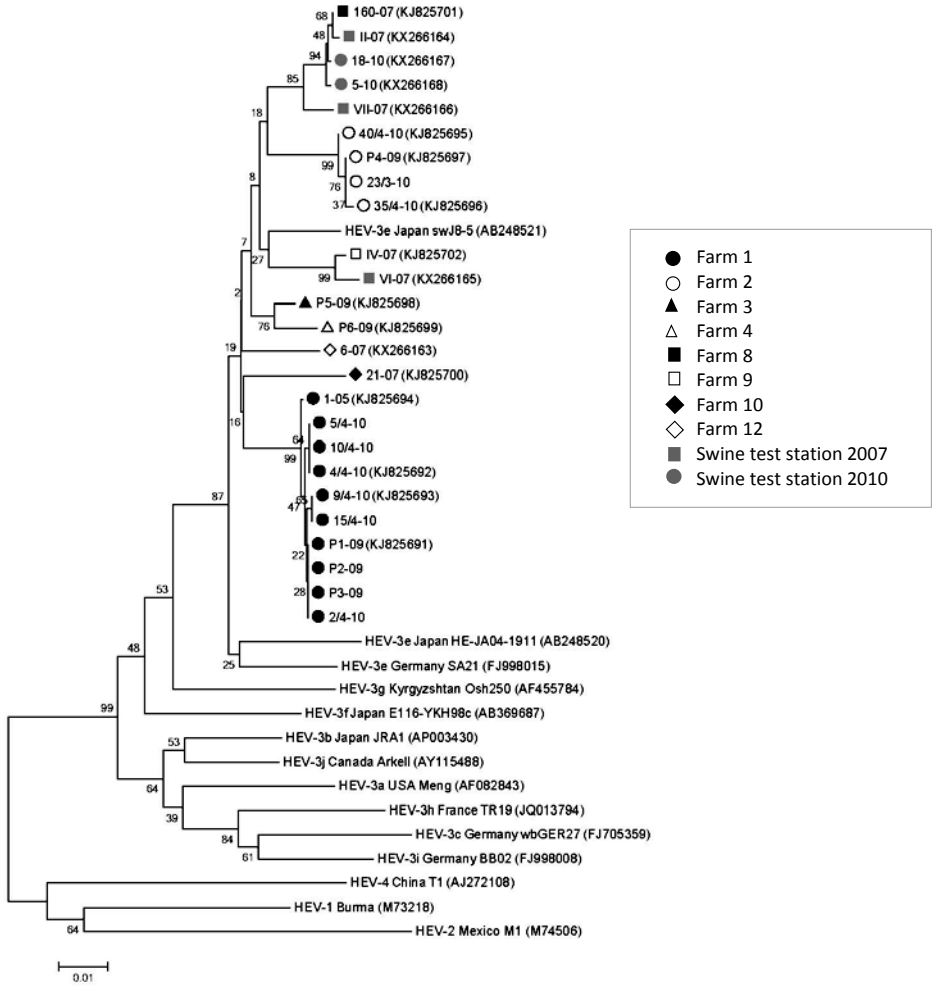

Figure 3. Phylogenetic tree based on a 268-nt fragment within the structural gene in ORF2, showing the 25 isolates from eight farms and the swine test station in 2007 and 2010 from this study, together with 14 previously reported HEV isolates of HEV-1, HEV-2, HEV-4, and HEV-3 subtypes a–c and e–j as proposed for reference sequences by Smith et al. (2016).

5.5 Comparison of sensitivity and suitability of two sets of primers and conventional RT-PCR methods targeting different regions of the HEV genome for sequencing and genetic characterization of swine HEV (II, unpublished)

The real-time RT-PCR, genotyping RT-PCR, and sequencing results of 61 swine fecal samples from Stydy II were included in comparison of sensitivity and suitability of a one-step RT-PCR method targeting the ORF1 region, and a nested RT-PCR method targeting the ORF2 region of the HEV genome for sequencing and genetic characterization of swine HEV (Table 12). From 30 (49%) of these samples, a total of 43 sequences were obtained. The ORF1-targeting one-step RT-PCR method yielded more (44%) positive results than the ORF2-targeting nested RT-PCR (28%). In total, 51% (31/61) of the HEV RNA-positive samples were positive by either one or both of the RT-PCR methods. An ORF1 sequence was obtained from all 27 samples that were positive by the ORF1-targeting primers and an ORF2 sequence from all but one (a sample with a very high C_t value of 44.4) of the 17 samples that were positive by the ORF2-targeting primers. Both ORF1 and ORF2 sequences were obtained from a total of 13 samples.

Of the HEV RNA-positive samples with C_t values of less than 30, 82% (9/11) yielded a sequence with both the ORF1- and the ORF2-targeting primers. As the C_t values increased in an RNA load-dependent manner, the number of sequences decreased; of the RNA-positive samples with C_t values 30 and above, but less than 40, 55% (16/29) gave a sequence by the ORF1-targeting one-step RT-PCR, and only less than half of that amount, 24% (7/29), by the ORF2-targeting nested RT-PCR. In total, the sensitivities of 44% for the ORF1 and of 28% for the ORF2-targeting RT-PCR showed a higher detection rate of HEV for sequencing and genotyping when the ORF1-targeting method was used.

Table 12. Results of genotyping RT-PCRs and sequencing with the ORF1-targeting and ORF2-targeting primers of the swine fecal samples found HEV RNA-positive by real-time RT-PCR, according to ascending C_t values.

	Result in genotyping RT-PCR and sequencing by the two primer sets			
C_t value in real-time RT-PCR	Number of ORF1 positive samples (%)	Number of ORF2 positive samples (%)	Number of negative samples (%)	Total number of samples tested
20– < 30	9 (82)	9 (82)	0 (0)	11
30– < 35	16 (55)	7 (24)	12 (41)	29
35–40	2 (14)	0 (0)	12 (86)	14
> 40	0 (0)	1[1] (14)	6 (86)	7
Total	27 (44)[2]	17[1, 2] (28)	30 (49)	61

[1]No sequence was obtained from one sample despite a positive band in the gel with the ORF2-targeting primers; the total number of ORF2 sequences obtained was 16.
[2]In total, 43 sequences were obtained from 30 samples; both an ORF1 sequence and an ORF2 sequence were obtained from 13 samples.

The ORF1 sequences formed eight separate clusters and the ORF2 sequences seven clusters. At the nucleotide level, the maximum difference of the ORF1 sequences within these clusters was 2.3% (5/216 nt), and the maximum difference of the ORF2 sequences, 0.8% (2/268 nt). Among all clusters, the differences in the sequences were 6.0–12.6% with the ORF1 primers and 3.4–11.6% with the ORF2 primers.

On the amino acid level, all of the ORF2 sequences were 100% identical. Between the ORF1 sequences, variation of 1–3 amino acids in reference to the HEV prototype Burma strain (M73218) was observed at positions 1440 (serine or proline), 1461 (glycine or valine), and 1462 (threonine or serine).

5.6 Presence of anti-HEV antibodies in Finnish veterinarians and evaluation of possible work-related risk factors associated with HEV exposure in veterinarians (IV)

Of the veterinarians investigated, 10.2% (34/333) were positive for anti-HEV total antibodies. Of the non-veterinary participants of the study, 5.8% (3/52) were seropositive. Anti-HEV IgM antibodies were not detected in any of the samples that were anti-HEV total antibody-positive. However, one anti-HEV IgM-positive, but HEV RNA-negative sample was detected among the 93 randomly selected anti-HEV total antibody-negative samples that were investigated.

No significant difference in total HEV antibody seropositivity existed between veterinarians and non-veterinarians or between the sexes. Most of the seropositive subjects were females: 79.4% among veterinarians, 100% among non-veterinarians, and 81.1% among all subjects. The HEV-seropositive subjects were aged between 25 and 79 years. Among veterinarians, seropositive females were aged between 25 and 69 years, and seropositive males between 45 and 79 years, and the two seropositive non-veterinarians who had provided their age were 45 and 46 years. The proportional number of HEV-seropositive subjects increased steadily with age from 1.6% among 20- to 29-year-olds to 25.0% among 50- to 59-year-olds.

Among veterinarians, a significantly higher seroprevalence of 17.8% (13/73) was detected among small animal practitioners than the seroprevalence of 8.5% (14/165) among municipal veterinarians or 3.6–8.7% among other job specialties (p=0.01) (Table 13).

Table 13. Distribution of subjects tested and subjects positive for anti-HEV total antibodies according to occupation (veterinarian/non-veterinarian) and the job specialty categories of the veterinarians.

Occupation (non-veterinarian/ veterinarian) and job specialty categories ((1)–(8)) of the veterinarians	Number of subjects tested in the occupational group (% of all subjects[1]) or job category (% of veterinarians[2])	Number of HEV-seropositive subjects in the occupational group or job specialty category (%; 95% CI)	Number of HEV-seropositive subjects/number of subjects tested not in the occupational group or job specialty category (%; 95% CI)	*p*
Non-veterinarian	52[1] (13.5)	3 (5.8; 1.4–16.3)	34/333 (10.2; 7.4–14.0)	0.33
Veterinarian	333[1] (86.5)	34 (10.2; 7.4–14.0)	3/52 (5.8; 1.4–16.3)	
(1) Municipal veterinarian[3]	165[2] (53.2)	14 (8.5; 5.0–13.8)	17/145 (11.7; 7.4–18.1)	0.35
(2) Small animal practitioner only	**73[2] (23.5)**	**13 (17.8; 10.6–28.3)**	**18/237 (7.6; 4.8–11.8)**	**0.01**
(3) Slaughterhouse[3]	46[2] (14.8)	4 (8.7;2.9–20.9)	27/264 (10.2; 7.1–14.5)	0.79
(4) Horse clinic[3]	12[2] (3.9)	1 (8.3;0.0–37.5)	30/298 (10.1; 7.1–14.0)	0.93
(5) Laboratory[3]	40[2] (12.9)	3 (7.5; 1.9–20.6)	28/270 (10.4; 7.2–14.6)	0.61
(6) Office only	16[2] (5.2)	1 (6.3; 0.0–30.3)	30/294 (10.2; 7.2–14.2)	0.69
(7) Other	28[2] (9.0)	1 (3.6; 0.0–19.2)	30/282 (10.6; 7.5–14.8)	0.25
(8) Data not provided	6[2] (1.9)	1 (16.7; 1.1–58.2)	30/304 (9.9; 7.0–13.8)	0.58

Statistically significant *p* values ($p < 0.05$) are in bold.

CI, confidence interval.

[1]In total, 385 samples were tested, 52 from non-veterinarians and 333 from veterinarians.

[2]For further statistical analyses of the job specialty categories (1)–(8) of the veterinarians, samples from 310 veterinarians were included, samples from 23 veterinarians who did not complete the electronic questionnaire and who therefore did not provide further data were excluded.

[3]All veterinarians who reported belonging to the job category, only or in addition to other job specialty categories.

The selected possible risk factors for zoonotic HEV exposure analysed are presented in Table 14. Approximately three of four veterinarians (74.5%) reported having had needle stick by an injection needle that had previously been injected into any animal, and 7.1% reported having had a needle stick by an injection needle that had previously been injected into a pig. Work-related contacts with swine and wild boars during the five preceding years had been experienced by 71.0% and 25.1% of the veterinarians, respectively. HEV seropositivity was negatively associated with having had contact with swine during the preceding five years among all veterinarians (6.4% and 18.9% with and without swine contacts, respectively; p=0.002) and among small animal practitioners (6.1% and 27.5% with and without swine contacts, respectively; p=0.02). However, conflictingly, the seroprevalence appeared to be higher in those who had experienced needle stick by a needle that had previously been injected into a pig than in those who had not (difference not statistically significant) among all veterinarians as well as among small animal practitioners.

Of the non-veterinarians, 29.8% (14/47) reported having had contact with swine and 10.6% (5/47) with wild boars during the previous five years, but none were found to be seropositive. A majority of all participants, 88.5% (316/357), ate pork. Of them, 8.9% (28/316) were positive for anti-HEV total antibodies, which did not differ significantly from the seroprevalence of those who did not eat pork. Most, 95% (339/357), of all participants reported travels abroad during the preceding five years: 93% outside Scandinavia and 62.7% outside Europe. Only 4.2% had visited HEV-1 or HEV-2 endemic areas in Asia, Africa, or Mexico during the preceding six-month period. Thirty-two participants (9.0%) reported having received hospital care abroad. Although none of these factors were recognized as risk factors for the presence of anti-HEV total antibodies, the seroprevalence appeared to be higher in those small animal practitioners who had traveled outside Europe than in those who had not (difference not statistically significant, p=0.06).

Table 14. Numbers of tested veterinarians and tanti-HEV total antibody positive veterinarians with and without selected possible risk factors for HEV exposure.

		Risk factor					
Veterinary job specialty category		Contacts with swine[1]	A lot of contacts with swine[1]	Contacts with wild boars[1]	A lot of contacts with wild boars[1]	Needle stick by a needle that had previously been injected to an animal	Needle stick by a needle that had previously been injected to a pig
All veterinarians (N=310[2])	N of tested with (% of all)/ without risk factor	220 (71.0)/90	96 (31.0)/214	80 (25.8)/230	2 (0.6)/308	231 (74.5)/79	22 (7.1)/288
	N of positive with risk factor (%; 95% CI)	14 (6.4; 3.7–10.5)	6 (6.3; 2.6–13.2)	8 (10.0; 4.9–18.8)	1 (50.0; 9.5–90.6)	26 (11.3; 7.8–16.0)	5 (22.7; 9.7–43.9)
	N of positive without risk factor (%; 95% CI)	17 (18.9; 12.1–28.3)	25 (11.7; 8.0–16.7)	23 (10.0; 6.7–14.6)	30 (9.7; 6.9–13.6)	5 (6.3; 2.4–14.3)	26 (9.0; 6.2–12.9)
	p	**0.002**	0.14	0.98	0.20	0.21	0.07
(1) Municipal veterinarian[3] (N=165)	N of tested with (% of all)/ without risk factor	141 (85.5)/24	68 (41.2)/97	55 (33.3)/110	1 (0.6)/164	142 (86.1)/23	16 (9.7)/149
	N of positive with risk factor (%; 95% CI)	10 (7.1; 3.8–12.7)	4 (5.9; 1.9–14.6)	6 (10.9; 4.7–22.2)	1 (100; 16.8–100)	12 (8.5; 4.8–14.3)	3 (18.8; 5.8–43.8)
	N of positive without risk factor (%; 95% CI)	4 (16.7; 6.1–36.5)	9 (9.3; 4.8–16.9)	8 (7.3; 3.5–13.9)	13 (7.9; 4.6–13.2)	2 (8.7; 1.2–28.0)	11 (7.4; 4.0–12.9)
	p	0.16	0.45	0.44	0.08	0.92	0.17
(2) Small animal practitioner only (N=73)	N of tested with (% of all)/ without risk factor	33 (45.2)/40	8 (11.0)/65	10 (13.7)/63	0 (0.0)/73	58 (79.5)/15	3 (4.1)/70
	N of positive with risk factor (%; 95% CI)	2 (6.1; 0.7–20.6)	1 (12.5; 0.1–49.2)	1 (10.0; 0.0–42.6)	0 (0.0; 0.0–100)	12 (20.7; 12.1–32.9)	2 (66.7; 20.3–94.4)
	N of positive without risk factor (%; 95% CI)	11 (27.5; 16.0–43.0)	12 (18.5; 10.7–29.7)	12 (19.1; 11.1–30.6)	13 (17.8; 10.6–28.3)	1 (6.7; 0.0–31.8)	11 (15.7; 8.8–26.2)
	p	**0.02**	0.75	0.55	ND	0.23	0.08

Table 14. *(Continued)*

Veterinary job specialty category		Risk factor					
		Contacts with swine[1]	A lot of contacts with swine[1]	Contacts with wild boars[1]	A lot of contacts with wild boars[1]	Needle stick by a needle that had previously been injected to an animal	Needle stick by a needle that had previously been injected to a pig
(3) Slaughter-house[3] (N=46)	N of tested with (% of all)/without risk factor	38 (82.6)/8	23 (50.0)/23	18 (39.1)/28	0 (0.0)/46	33 (71.7)/13	5 (10.9)/41
	N of positive with risk factor (%; 95% CI)	3 (7.9; 2.0–21.5)	1 (4.3; 0.0–22.7)	2 (11.1; 1.9–34.0)	0 (0.0; 0.0–100)	3 (9.1; 2.4–24.3)	0 (0.0; 0.0–48.9)
	N of positive without risk factor (%; 95% CI)	1 (12.5; 0.1–49.2)	3 (13.0; 3.7–33.0)	2 (7.1; 0.9–23.7)	4 (8.7; 2.9–20.9)	1 (7.7; 0.0–35.4)	4 (9.8; 3.3–23.1)
	p	0.68	0.36	0.67	ND	0.94	0.62
(4) Horse clinic[3] (N=12)	N of tested with (% of all)/without risk factor	8 (66.7)/4	5 (41.7)/7	6 (50.0)/6	0 (0.0)/12	12 (100)/0	0 (0.0)/12
	N of positive with risk factor (%; 95% CI)	0 (0.0; 0.0–37.2)	0 (0.0; 0.0–48.9)	0 (0.0; 0.0–44.3)	0 (0.0; 0.0–100)	1 (8.3; 0.0–37.5)	0 (0.0; 0.0–100)
	N of positive without risk factor (%; 95% CI)	1 (25.0; 3.4–71.1)	1 (14.3; 0.5–53.4)	1 (16.7; 1.1–58.2)	1 (8.3; 0.0–37.5)	0 (0.0; 0.0–100)	1 (8.3; 0.0–37.5)
	p	0.33	0.58	0.50	ND	ND	ND
(5) Labora-tory[3] (N=40)	N of tested with (% of all)/without risk factor	32 (80.0)/8	13 (32.5)/27	14 (35.0)/26	1 (2.5)/39	31 (77.5)/9	3 (7.5)/37
	N of positive with risk factor (%; 95% CI)	2 (6.3; 0.7–21.2)	1 (7.7; 0.0–35.2)	2 (14.3; 2.8–41.2)	1 (100; 16.8–100)	3 (9.7; 2.6–25.7)	1 (33.3; 5.6–79.8)
	N of positive without risk factor (%; 95% CI)	1 (12.5; 0.1–49.2)	2 (7.4; 1.0–24.5)	1 (3.8; 0.0–20.5)	2 (5.1; 0.5–17.8)	0 (0.0; 0.0–34.5)	2 (5.4; 0.6–18.6)
	p	0.59	0.95	0.31	0.08	0.46	0.22
(6) Office only (N=16)	N of tested with (% of all)/without risk factor	6 (37.5)/10	1 (6.3)/15	0 (0.0)/16	0 (0.0)/16	6 (37.5)/10	0 (0.0)/16
	N of positive with risk factor (%; 95% CI)	0 (0.0; 0.0–44.3)	0 (0.0; 0.0–83.3)	0 (0.0; 0.0–100)	0 (0.0; 0.0–100)	1 (16.7; 1.1–58.2)	0 (0.0; 0.0–100)
	N of positive without risk factor (%; 95% CI)	1 (10.0; 0.0–42.6)	1 (6.7; 0.0–31.8)	1 (6.3; 0.0–30.3)	1 (6.3; 0.0–30.3)	0 (0.0; 0.0–32.1)	1 (6.3; 0.0–30.3)
	p	0.63	0.94	ND	ND	0.38	ND

Table 14. *(Continued)*

Veterinary job specialty category		Risk factor					
		Contacts with swine[1]	A lot of contacts with swine[1]	Contacts with wild boars[1]	A lot of contacts with wild boars[1]	Needle stick by a needle that had previously been injected to an animal	Needle stick by a needle that had previously been injected to a pig
(7) Other (N=28)	N of tested with (% of all)/without risk factor	21 (75.0)/7	10 (35.7)/18	8 (28.6)/20	1 (3.6)/27	9 (32.1)/19	2 (7.1)/26
	N of positive with risk factor (%; 95% CI)	1 (4.8; 0.0–24.4)	0 (0.0; 0.0–32.1)	0 (0.0; 0.0–37.2)	0 (0.0; 0.0–83.3)	0 (0.0; 0.0–34.5)	0 (0.0; 0.0–71.0)
	N of positive without risk factor (%; 95% CI)	0 (0.0; 0.0–40.4)	1 (5.6; 0.0–27.6)	1 (5.0; 0.0–25.4)	1 (3.7; 0.0–19.8)	1 (5.3; 0.0–26.5)	1 (3.8; 0.0–20.5)
	p	0.75	0.64	0.71	0.96	0.68	0.93
(8) Data not provided (N=6)	N of tested with (% of all)/without risk factor	2 (33.3)/4	0 (0.0)/6	1 (16.7)/5	0 (0.0)/6	2 (33.3)/4	1 (16.7)/5
	N of positive with risk factor (%; 95% CI)	0 (0.0; 0.0–71.0)	0 (0.0)	0 (0.0; 0.0–83.3)	0 (0.0)	0 (0.0; 0.0–71.0)	0 (0.0; 0.0–83.3)
	N of positive without risk factor (%; 95% CI)	1 (25.0; 3.4–71.1)	1 (16.7; 1.1–58.2)	1 (20.0; 2.0–64.0)	1 (16.7; 1.1–58.2)	1 (25.0; 3.4–71.1)	1 (20.0; 2.0–64.0)
	p	0.67	ND	0.83	ND	0.67	0.83

N, number; CI, confidence interval; ND, not determined.
Statistically significant *p* values ($p < 0.05$) are in bold.
[1]Contacts with swine and wild boars during the five year period preceding the survey.
[2]Of the total of 333 samples from veterinarians tested, samples from 310 veterinarians who completed the electronic questionnaire and provided further data were included in the analysis.
[3]All veterinarians who reported belonging to the job specialty category, only or in addition to other job specialty categories.

5.7 Comparison of seroprevalences obtained by ELISA tests detecting anti-HEV total antibodies and anti-HEV IgG antibodies in human serum samples (IV, unpublished)

Thirty-seven samples tested anti-HEV total antibody positive with the Axiom assay in Study IV. Of these, 21 (57%) were positive for anti-HEV IgG antibodies with the *recom*Well assay (Table 15). In addition, 93 anti-HEV total antibody-negative samples from study IV were randomly selected in the analysis for anti-HEV IgG antibodies. All of these 93 anti-HEV total antibody-negative samples were also anti-HEV IgG-negative. The proportion of IgG-positive samples was highest at 88% (15/17) among samples that had OD values above 3 for the Axiom assay. In contrast, only 40% (6/15) of the samples with OD values between 1 and 2.999 and none of the samples with OD values below 1 in the Axiom assay were positive for the *recom*Well IgG assay.

Table 15. Results of the 130 samples included in the comparison of ELISA assays: the results of the anti-HEV IgG assay (recomWell) compared with the results of the anti-HEV total antibody assay (Axiom).

	Positive samples						
	OD from anti-HEV total antibody assay						
	< 1	1–1.999	2–2.999	3–3.999	≥ 4	Total number of positive samples	Number of negative samples
Number of samples (%)	5 (14)	4 (11)	11 (30)	5 (14)	12 (32)	37 (29)	93 (72)
Number of positive samples by anti-HEV IgG assay (%)	0	1 (25)	5 (46)	5 (100)	10 (83)	21 (57)	0

OD, optical density.

6 Discussion

6.1 Hepatitis E infections in human patients with unexplained acute hepatitis (I)

Both anti-HEV IgM and IgG antibodies, indicating an acute infection, were detected in 11.5% (11/96) of the patients with unexplained, non-A–C hepatitis, and also HEV RNA, confirming the infection, in 8 (72.7%) of them. Only IgG antibodies, indicating an earlier infection, were also found in 11.5% (11/96) of the patients. The prevalence of acute infections was similar to those detected in Italy and Spain in comparable studies on patients with unexplained hepatitis: in Spain, IgM antibodies were present in 11.4% of 277 patients, with HEV RNA detected in 61% of the IgM-positive cases (Echevarría et al., 2011), and in Italy, 10.1% of 218 patients were diagnosed with acute infections (Zanetti et al., 1999). Somewhat lower prevalences have been reported in other similar European studies.

In the Netherlands, evidence of acute HEV infection as the presence IgM antibodies was found in 3.3% of the 209 examined cases by Waar and colleagues (2005), and as the presence of both IgM and IgG antibodies in 4.4% of the 1027 patients examined in the study by Herremans and colleagues (2007b), in which detection of HEV RNA confirmed the diagnosis in 51% of the cases. Only IgG antibodies were detected in 6.2–6.7% of the cases by Waar and colleagues (2005), and in 4.2% of the cases by Herremans and colleagues (2007b). In Hungary, IgG antibodies were detected in 7.2% of 264 examined patients (Haagsman et al., 2007). In contrast, a clearly higher prevalence of 20.6% of acute infections with both IgM and IgG antibodies present was detected among 651 patients in Italy (Romanò et al., 2011). The differences between these prevalences can at least partly be explained by different sizes of study groups used.

All five HEV sequences obtained from human patients in this study belonged to genotype HEV-1, the most common genotype in the traditional HEV endemic areas in Asia, Africa, and Mexico (Lu et al., 2006; Purcell and Emerson, 2008). Three of the patients with sequenced samples had recently visited these areas, which are the likely sources of their infections. Although traveling history was unavailable for the other two patients with HEV-1 viruses, their infections can also be assumed to have originated from abroad. This assumption can also be made for one patient who had been to Spain and whose two samples were positive for both IgM and IgG antibodies. The effect of traveling outside Europe was demonstrated in a study by Norder and colleagues (2009), which was conducted in Sweden and Denmark on patients who were selected based on recent traveling history and having clinical signs of hepatitis not caused by hepatitis viruses A, B, C, or D. In that study, distinctly higher seroprevalences than those reported for patients who were not specifically selected based on traveling history in the other above-mentioned studies were reported; 54% of the patients in Sweden and 61% in Denmark had both IgM and IgG antibodies against HEV. Of sequenced HEVs in that study, 89% were of HEV-1, and the traveling histories indicated that those infections originated outside Europe. In the study by Zanetti and colleagues (1999) in Italy, 77.3% of the patients with acute infections had developed symptoms of hepatitis after returning from HEV endemic areas, including India, Pakistan, Bangladesh, and Somalia, and traveling in the endemic areas was demonstrated to be the main risk factor for HEV infections.

The results indicate that HEV must be considered a possible causative agent of non-A–C hepatitis in Finland, as in several other European countries, especially when the patient has recently visited an HEV-1 or HEV-2 endemic area. In this study, no infections acquired in Finland or caused by HEV-3 were detected. Since HEV-3 infections are usually asymptomatic or only mildly symptomatic (Lin et al., 2000; Mizuo et al., 2005; Ohnishi et al., 2006; Purcell and Emerson, 2008), the group of patients with acute hepatitis symptoms may have been suboptimal for finding HEV-3 infections. Furthermore, it is not certain whether the ELISA test used in this study was able to detect HEV-3 since commercial ELISA tests for HEV antibodies have been developed for use in endemic areas with high prevalence of infections (Zhang et al., 2002; Daniel et al., 2004; Mizuo et al., 2005; Waar et al., 2005). However, since 11.5% of the patients were positive for only IgG antibodies, indicating an earlier infection, the possibility of cases acquired in Finland cannot be overlooked.

6.2 HEV in production swine in Finland (II, III, unpublished)

6.2.1 Occurrence and dynamics of hepatitis E infections in production swine and presence of HEV at swine farms (II, III, unpublished)

An apparent overall age-related pattern of hepatitis E infections in pigs was observed in the studies. The youngest individual pigs shedding HEV RNA in their feces were detected at the age of 2–3 months, at weaner stage, the production stage at which the HEV RNA positivity rate was highest, 34.6%. After that, the positivity rate decreased to 21.1% at grower stage (age 3–4 months) and 2.9% at finisher stage (age ≥ 5 months). The results of HEV RNA detection from pooled fecal samples also showed that the HEV RNA positivity rate was highest among weaner pigs and grower pigs. However, the positivity rate was highest, 52.8%, among grower pigs, and lower, but still at the same rate as in individual pigs, 33.3%, among weaner pigs. No positive pooled samples from finisher pigs were observed, but these samples were only collected from the swine test station in 2007. The age-related pattern of HEV excretion was in line with other studies, which have demonstrated that fecal HEV excretion in pigs is most common at the age of 2–4 months (Cooper et al., 2005; Fernández-Barredo et al., 2006; Leblanc et al., 2007; Di Bartolo et al., 2008, 2011; McCreary et al., 2008; Breum et al., 2010; Feng et al., 2011; Berto et al., 2012a; Caruso et al., 2016).

The course of hepatitis E infections in pigs was observed in follow-up studies conducted on pigs at farrowing stage in Study II and at fattening stage in Study III. In Study II, the piglets at two farrowing farms began shedding HEV in their feces at weaner stage (age 7–12 weeks), 3–8 weeks after weaning, which took place at the ages of 3–4 weeks, after which the pigs were moved to nursery units where they were raised until transfer to fattening farms. In pigs, fecal excretion of HEV usually starts 1–2 weeks after infection (Meng et al., 1998; Halbur et al., 2001; Kasorndorkbua et al., 2004), which suggests that the first piglets in this study were most probably infected shortly, 1–2 weeks, after weaning. Although 25% of the piglets started to shed HEV at the age of 7–8 weeks, 63% of them only began shedding the virus at the age of 10–12 weeks.

In other longitudinal studies of fecal HEV excretion in pigs, comparable patterns of HEV excretion have been described. In Slovenia, the rate of RNA-positive pigs was 5.3% at sucker age, 28.6% at weaner age, and 26.9% at fattening stage (Steyer et al., 2011). In Spain, HEV RNA was detected in the feces of pigs from the age of 9 weeks onwards, most often (in 5/5 pigs investigated) at the age of 3–4 months (de Deus et al., 2008a). Earlier onsets of fecal excretion of HEV have also been reported; in Japan, the first HEV RNA detection in feces of piglets was at the age of 30 days (Kanai et al., 2010), in Spain in pigs aged 3 weeks (Casas et al., 2011a), and in Canada in 2-week-old pigs (11.8%) (Leblanc et al., 2007). In the Canadian study, the detection rate then increased to 52.9% at the age of 2 months and reached a peak of 86.2% at the age of 4.5 months (Leblanc et al., 2007). A possible reason for the varying age of onset of the infection and shedding of the virus is the quantity and/or quality of colostrum providing maternal antibodies to the piglets. The amount of sow antibodies affects the duration of a piglet´s passive immunity to HEV (Meng et al., 1997; de Deus et al., 2008a; Kanai et al., 2010), the duration of which is approximately 30 days after birth, but in some piglets, especially those born to strongly seropositive sows, it can last up to 60 days or even longer (Meng et al., 1997; Kasorndorkbua et al., 2003; de Deus et al., 2008a; Kanai et al., 2010; dos Santos et al., 2009; Feng et al., 2011). At the time of transfer to fattening farms, practically all pigs at the two farrowing farms examined in Study II had been infected by HEV and they were shedding the virus, enabling HEV transmission from farrowing farms to fattening farms.

In Study III, at the beginning of follow-up during the fattening period at the swine test station, at the age of 2–3 months, 22.4% (17/76) of the pigs examined were shedding HEV in their feces: 35% (14/40) in 2007 and 8.3% (3/36) in 2010. The rates of shedding at this point were lower than those observed at the end of the farrowing period in Study II. The difference is probably explained by the different farms from which the pigs originated. During the farrowing period, HEV infections were followed in piglets at two farms, at which HEV-positive pigs had previously been detected, whereas during the fattening period, pigs that originated from 11 farms in 2007 and from eight farms in 2010 were followed at the test station. In the beginning of the follow-ups during the fattening period, in 2007, HEV RNA-positive pigs from seven farms were detected, and all pigs from four farms were negative, whereas in 2010, positive pigs from two farms were detected, and pigs from six farms were all negative.

In the overall study, HEV RNA or anti-HEV antibodies were detected in samples collected from 56.8% of separate farms investigated. HEV RNA was found in samples collected from 50.0% of the farms examined. This suggests that there are both HEV-positive and -negative swine farms in Finland. Other studies in Europe have reported a vast variety of farm-level prevalence of HEV RNA in swine feces. In Italy, prevalences of 0–48.4% (Costanzo et al., 2015), 31% (Caruso et al., 2016), and 100% (Di Bartolo et al., 2008) have been detected in different studies. A prevalence of 38.1% has been reported in Spain (Fernández-Barredo et al., 2006), 33.3% in Slovenia (Steyer et al., 2011), 55% both in the Netherlands (Rutjes et al., 2007) and in Denmark (Breum et al., 2010), 63.6% in the Czech Republic (Vasickova et al., 2009), and 100% in the UK (McCreary et al., 2008). The differences are most probably due to the different ages of the pigs sampled and the different numbers of farms investigated in these studies.

Pig-to-pig transmission of HEV was observed in the follow-ups during the fattening period in Study III. New pigs shedding HEV RNA in their feces were detected after the first sampling of the follow-up conducted in 2010, and the pigs that were not positive until after 2–4 weeks at the swine test station must have been infected at the station. In two pens, no positive pigs were detected at

the beginning of the fattening period, but were detected only two weeks later, suggesting that the virus was transmitted between adjacent pens because the wall separating them allowed pigs to have snout contact. Andraud and colleagues (2013) demonstrated HEV transmission between pig pens via nasal secretions, although it was rare. In the study by Kasorndorkbua and colleagues (2004), transmission via nasal secretions was not shown. However, fecal material in the snout would create a fecal-oral route of transmission via snout contact. Nevertheless, the possibility to trace the spread of HEV by sequences was limited in this study, because a sequence was only obtained from 60% of the HEV RNA-positive samples, most probably due to different sensitivities of the RT-PCR methods used. The real-time RT-PCR method used for detection was more sensitive than the conventional RT-PCR method used for sequencing, and thus, weaker positive samples that were detected by the real-time RT-PCR were not detected by the less sensitive conventional RT-PCR.

No HEV RNA-positive pigs at finisher age (5–6 months) just before slaughter were detected in the follow-ups in Study III. However, in the overall study, 2.9% (6/210) of samples from slaughter-aged pigs were found HEV RNA-positive. Of the pigs with serum samples available, 84% were positive for anti-HEV total antibodies at the time of slaughter in Study III. Since only 43% of the seropositive pigs were HEV RNA-positive during the follow-ups in Study III, 57% potentially carried the infection at the farrowing farms, before entering the test station. The presence of existing antibodies at the beginning of the fattening period would also explain why no new infections were detected during the 2007 follow-up. Another possibility is that in some cases fecal shedding of the virus was not detected due to long (4–6 weeks) intervals between consecutive samplings in 2007, since although pigs may excrete HEV RNA in their feces for up to 7–11 weeks (Meng et al., 1998; Halbur et al., 2001; Kasorndorkbua et al., 2004; Kanai et al., 2010), fecal shedding lasting only 11–17 days has also been reported in contact-infected pigs (Bouwknegt et al., 2008b). In the 2010 follow-up, however, the sampling interval was two weeks, and thus, it was unlikely that infections were overlooked.

Diverse rates of HEV RNA in the feces of slaughter-aged pigs have been reported in other studies, ranging from 0% (Kaba et al., 2009; Kanai et al., 2010; Casas et al., 2011a) to 7.0–41.2% (Fernández-Barredo et al., 2006; Nakai et al., 2006; Leblanc et al., 2007, 2010; Di Martino et al., 2010; Di Bartolo et al., 2011; Berto et al., 2012a, 2012c; Gardinali et al., 2012). The overall seroprevalence of 87.5% in slaughter-aged pigs in this study was comparable with the seroprevalence of 88% recently reported in Corsica, France (Jori et al., 2016), but high relative to other seroprevalences reported in Europe, most of which have been between 60% and 70%: 61.4% in Scotland (Crossan et al., 2015), 64% in Spain (Casas et al., 2011b), and 68.6% in Germany (Wacheck et al., 2012). A lower prevalence of 31% was found in France (Rose et al., 2011). In another study from France, an overall seroprevalence of almost 75% was detected, but a variation range of 0–40% was observed between farms (Walachowski et al., 2013). In Canada, the overall seroprevalence was 59.4%, but regional differences raging between 38.3% and 88.8% were noted (Yoo et al., 2001). A high seroprevalence of 96.9% has been reported in China (Feng et al., 2011), whereas the seroprevalence was only 25% in Korea (Choi et al., 2003). Thus, variation is present in HEV seroprevalence of slaughter-aged pigs depending on the county, the geographical areas within countries, the study size, and the study settings.

Overall, as expected, HEV RNA was most often detected in pigs aged 2–4 months. The results suggest that pigs acquire HEV infection either at farrowing farms shortly after weaning or at the

beginning of their growing period at fattening farms, and most of them are cleared from the infection before slaughter. The HEV seropositivity rate remained high, over 80%, from weaners to sows, demonstrating that the majority of pigs in Finland are infected with HEV during their lives.

6.2.2 Genetic divergence of HEV strains occurring in production swine at different swine farms (II, III, unpublished)

All HEV isolates obtained from swine in studies II and III, and in the unpublished swine studies belonged to genotype HEV-3 and within it, subtype e. The ORF1 primers amplified a fragment of the RNA polymerase gene of HEV at the 3´ end of ORF1. Within the eight clusters of sequences obtained, there was a maximum difference of 5.1% between sequences, and between the clusters, the nucleotide difference was 6.1–12.5%. Lu et al. (2006) determined that based on that same area of the HEV genome on the nucleotide level, there was a difference of 7.0–12.1% between HEV isolates, whereas the difference between HEV subtypes was 11.4–22.8%. Somewhat lower difference of 9.4–17.2% between HEV-3 subtypes e, f, and g was determined by Doceul et al. (2016). The highest difference, 12.5%, between clusters from this study was above the percentage difference determined for isolates by Lu et al. (2016), but according to phylogenetic analysis, this cluster clearly belonged to the same subtype 3e as the other clusters, and did not form a separate subtype. The ORF2 primers targeted the structural gene of HEV in the middle region of ORF2, and the maximum difference between the sequences within the seven clusters was 4.9%, whereas the difference between the clusters was 5.6–13.5%. According to Lu et al. (2006), the difference between isolates was 2.0–10.1% at the 5´ end and 5.6–14.8% at the 3´ end of ORF2, whereas the difference between subtypes was 12.6–19.8% and 12.9–19.3%, respectively. Thus, when the percentage nucleotide differences between the clusters of sequences from this study were interpreted according to the differences determined for isolate level by Lu et al. (2016), each of the clusters of both ORF1 and ORF2 sequences formed an isolate of their own, suggesting that a separate HEV isolate originated from five of the 11 separate swine farms from which sequences were obtained. From one of these farms sequences from the span of six years (2005–2010) all belonged to the same isolate. To the author´s knowledge, a long follow-up of genetic differences of HEV isolates at the same farm has not been reported elsewhere. In contrast, two different isolates originated from one separate farm in 2007 and from the swine test station in 2007.

Interestingly, all isolates originating from the samples collected from the swine test station in 2010 belonged to the same cluster, which also contained some isolates from the test station from 2007. It appeared that different isolates were introduced to the test station in 2007 by pigs arriving there from different farms. One of the isolates then persisted at the station or was brought there again in 2010 from several farms simultaneously. As a limitation of the study, the geographical distribution of the farms from which the piglet producers sent piglets to the station was not available, leaving the origins and possible geographical distribution of the different isolates unexplained.

Overall, the divergence of isolates originating from separate swine farms and from the swine test station on two occasions suggests that genetic variations in HEV strains originating from different locations occurred, although all strains belonged to the same subtype e of HEV-3. This suggests that subtype e is the main subtype circulating among production pigs in Finland. In several other European countries several different subtypes of HEV-3 appear. For example, at least three

subtypes, c, e, and f, have been isolated from swine in both Italy and the Netherlands, and two subtypes, c and f, in France (Martelli et al., 2010; Di Bartolo et al., 2011; Rose et al., 2011; Lapa et al., 2015).

As could be expected, more homogeneity was observed at the amino acid level between the sequences; all ORF2 sequences were identical with each other, whereas the ORF1 sequences only formed six separate groups, in contrast to the eight clusters formed by the nucleotide sequences. Most heterogeneity was seen among the amino acid sequences from farm 1, which were divided into four separate groups. Two sequences from 2005 differed by one amino acid from each other, whereas the difference between the 2005 sequences and the sequences from 2009–2010 was 2–3 amino acids, showing that although the same HEV strain was maintained at the farm over the years, a maximum of three amino acids had been replaced over time.

6.3 Comparison of sensitivity and suitability of two sets of primers and conventional RT-PCR methods targeting different regions of the HEV genome for sequencing and genetic characterization of swine HEV (II, unpublished)

Currently, no consensus exists regarding the genomic region that should be used for genotyping HEV. Recombination has been reported to be most common in the X domain, the RNA helicase, and the RNA-dependent RNA polymerase in ORF1 (Chen et al., 2012). The mean nucleotide distances over the HEV genome are relatively homogeneous, except for the area of the papain-like cysteine protease domain and the hypervariable region in ORF1, which peak in variability (Smith et al., 2013). In addition, reduced variability was found near the beginning of the HEV genome and in the region where ORF2 and ORF3 overlap (Smith et al., 2013).

Of the primer sets and RT-PCR methods compared in this study, the ORF1-targeting one-step RT-PCR method resulted in a higher sensitivity in detecting swine HEV for sequencing, and it was also faster and more practical to use than the ORF2-targeting nested RT-PCR method. Both the ORF1 and ORF2 regions of the HEV genome that were amplified by the primer sets compared in this study were representative of the full-length genome of HEV for genotyping and classification (Zhai et al., 2006; Xun et al., 2007). Zhai and colleagues (2006) found that the genomic region in the RNA-dependent RNA polymerase domain (nt 4254–4560) in ORF1 statistically substituted for the full-length genome of HEV for genotyping. The primer pair used in this study amplified a sequence that was 50 bp shorter at the 3′ end than the region described by Zhai and colleagues (2006), but was otherwise identical. Xun and colleagues (2007) verified that another region in the 5′ end of ORF2 (nt 5994–6294), which was amplified by the ORF2-targeting primers in this study, was even more representative of the full-length genome for HEV classification. The nucleotide differences observed in sequences from this study showed that the amplified genomic region of ORF2 was more conserved than the ORF1 region, both within and between the HEV-3 subtype e isolates. This was also observed at the amino acid level, since the ORF2 amino acid sequences were all identical, whereas variation was present among the ORF1 sequences. The variation at the nucleotide and amino acid levels did not affect the genetic classification of the isolates, but in investigations requiring source tracking of the virus, amplification and sequencing of the ORF1 region can be more beneficial. Although both the ORF1- and the ORF2-targeting RT-PCR

methods were equally sensitive in amplifying strongly positive samples for sequencing and genotyping, the ORF1-targeting method was more than twice as sensitive as the ORF2-targeting method when weaker positive samples were amplified. Thus, in aiming to sequence a sample with a C_t value higher than 30 in the real-time RT-PCR, the ORF1-targeting method would be preferable to yield a sequence for genotyping. Additionally, the one-step RT-PCR method used with the ORF1-targeted method was clearly more time-saving and more practical to use than the nested RT-PCR method used with the ORF2-targeted primers. Although whole-genome sequencing is increasingly used and is preferred for classification of HEV (Liu et al., 2008), there is still a need for sensitive partial-genome amplification methods, especially for samples in which the viral load is low, an important example being environmental samples.

6.4 Presence of anti-HEV antibodies in Finnish veterinarians and evaluation of possible work-related risk factors associated with HEV exposure in veterinarians (IV)

The 10.2% prevalence of anti-HEV total antibodies detected in Finnish veterinarians shows that antibodies against HEV are common in Finnish veterinarians, although the seroprevalence was lower than the HEV IgG seroprevalences reported for veterinarians elsewhere; in the USA, a seroprevalence of 21–23% (Meng et al., 2002) and in Germany 18.8% (Krumbholz et al., 2012) has been reported. Surprisingly, the highest seroprevalence of 17.8% was detected among small animal practitioners, compared with 8.5% among municipal veterinarians, most of whom visited farms and had contact with swine (89.1% and 85.5%, respectively). In the USA, seroprevalences of 30%, 23%, 22%, and 6% were reported in the veterinary job categories of industry, practicing, academic, and student, respectively (Meng et al., 2002). A seroprevalence of 9.9%, which was close to the seroprevalence of 8.5% detected among municipal veterinarians in this study, was reported for pet veterinarians in Portugal (Mesquita et al., 2014b).

Unexpectedly, a significantly higher proportion of seropositive veterinarians was observed among those who had no contact with swine (18.9%) than among those who had (6.4%), contrary to the results from the Netherlands, where 11% of swine veterinarians and 6% of non-swine veterinarians were positive for HEV IgG antibodies (Bouwknegt et al., 2008a). In France, a seroprevalence of 19.6%, which was close to the seroprevalence in those without swine contacts in this study, was reported for swine veterinarians (Chaussade et al., 2013). In addition, several previous studies on non-veterinary populations have shown that persons who have contact with swine have a higher risk for HEV infection than those who do not have contact with swine (Galiana et al., 2008; Krumbholz et al., 2012, 2014; Chaussade et al., 2013). However, a wide range of HEV seroprevalences in European countries has been reported for persons who come into contact with swine, from HEV IgG prevalence of 3.3% in pig breeders in Italy (Vulcano et al., 2007) and 13.4% in pig farm workers in Estonia (Ivanova et al., 2015) to HEV seroprevalence of 51.1% in swine farmers in Moldova (Drobeniuc et al., 2001). Moreover, 50.4% of all farmers were HEV antibody-positive in Denmark (Christensen et al., 2008). This shows that the variation occurs depending on the country and the population selected, in addition to the variety of diagnostic tests used for the studies, emphasizing the need to develop a gold standard for HEV antibody testing and comparison of test results, especially in regions with low seroprevalence.

There were also other factors that may have influenced the results, the main one being that all Finnish veterinarians have contacts with swine at some point during their careers, at least during their veterinary studies. HEV IgG antibodies have been reported in individuals for up to 23 years after they had an infection (Hogema et al., 2014). Of the 22 veterinarians who reported having had no contact with swine, four paradoxically stated having had needle stick by a needle that had previously been injected into a pig, and two of these individuals were seropositive. This suggests that some seropositive subjects who had not had contact with swine during the five years preceding the survey might have indeed contracted the infection from contact with swine that occurred even earlier. Additionally, the questionnaire did not contain specific questions about contacts with pet pigs or mini pigs, which may be treated by small animal practitioners, and from which a possible zoonotic infection has been reported (Renou et al., 2007).

Contradictory to swine contacts, the seroprevalence appeared to be higher in those who had had a needle stick by a needle that had previously been injected into a pig than in those who had not, 22.7% and 9.0%, respectively, which suggests that a possible contact with blood or tissue fluid from swine might be a risk factor for HEV infection. The seroprevalences among veterinarians who had and who had not reported a needle stick by a needle previously injected into an animal were 11.3% and 6.3%, respectively. The difference appears similar to the difference reported in the USA, where 25% and 15% of veterinarians who had and who had not reported needle stick were seropositive, respectively (Meng et al., 2002).

Traveling abroad was reported by the majority of the subjects (95.0%), and more specifically, more than half (62.5%) had traveled to non-European destinations during the five years preceding the survey. Traveling history may at least partly explain the high HEV seroprevalence detected among small animal practitioners since 23.1% of those who had traveled to non-European destinations were seropositive, in contrast to only 4.8% of those who had not, although the difference was not significant. Moreover, in general, the possibility of HEV infections being contracted abroad cannot be disregarded since traveling history during only the preceding five years was inquired, and HEV IgG antibodies remain detectable much longer (Hogema et al., 2014).

Although the difference in seroprevalence between veterinarians and non-veterinarians was not statistically significant, it is similar to the findings of several earlier studies conducted in Europe. In Sweden, HEV seroprevalence of 13.0% was reported for swine farmers and 9.2% for a control population (Olsen et al., 2006). In Germany, seroprevalences of 18.8% among veterinarians, 15.5% among blood donors (Krumbholz et al., 2012), and, depending on the diagnostic test used, 13.2–32.8% among individuals in contact with swine, and 7.7–21.7% among those without such contact (Krumbholz et al., 2014) have been reported. However, it is important to note that the group of non-veterinarians in this study could not be considered a proper control group since it included persons with some contact with animals and also at approximately 14% of the whole study population the number of non-veterinarians was small. In addition, the aim of this study was not to compare HEV seroprevalences between veterinarians and non-veterinarians, but to compare HEV exposure in veterinarians working in different practice specialties. Thus, further studies are required to define the HEV antibody prevalence difference between veterinarians and other occupational groups in Finland. The overall age-related pattern observed in this study whereby the HEV seroprevalence increased with age was in line with the results of previous studies (Meader et al., 2010; Carpentier et al., 2012; Rapicetta et al., 2013).

One HEV IgM-positive subject was found among those who tested negative for total HEV antibodies, and the sample was also negative for HEV IgG antibody and HEV RNA. It is known that IgM antibodies appear alone for a short time at the beginning of an acute HEV infection before IgG antibodies manifest, simultaneously with peak viremia (Kamar et al., 2012). However, this sole finding of HEV IgM antibodies, which might suggest an early phase of an acute infection, remained unconfirmed. Since no acute infections by the presence of HEV RNA that would have allowed the determination of the genotype of the HEV were found, it was not possible to confirm whether some of the infections could have been zoonotic or autochthonous either.

Overall, the results suggest that direct contact with swine is probably not the only route of HEV infection in veterinarians, and that multiple factors, including traveling outside Europe and reservoirs of HEV other than pigs, should be considered when investigating the sources of HEV infections for veterinarians. HEV seroprevalence in the general population in Finland has not yet been studied, and thus, investigation of whether Finnish veterinarians have a higher HEV seroprevalence than the general population is a future topic of interest. This would provide a basis for comparison to evaluate the significance of veterinary work as a risk factor for HEV infection in Finland.

6.5 Comparison of seroprevalences obtained by ELISA tests detecting anti-HEV total antibodies and anti-HEV IgG antibodies in human serum samples (IV, unpublished)

In comparison of the ELISA test results of the 130 samples selected from Study IV, 28.5% and 16.2% were positive for the Axiom total HEV antibody assay and the r*ecom*Well HEV IgG assay, respectively. The results resemble those from a previous study in which the same assays were compared, which yielded respective figures of 27.9% and 13.8% (Krumbholz et al., 2014). The expected sensitivity of the Axiom assay according to the manufacturer is 83%. A sensitivity of 96.3% and a specificity of 98.2% for the *recom*Well IgG assay in an acute infection setting are reported by its manufacturer. The Axiom assay detects total antibodies against HEV (IgM, IgG, and possibly also IgA). It is therefore logical that the Axiom assay yielded more positive results than the *recom*Well assay, as the latter detects only HEV IgG antibodies. The results of the comparison of the HEV ELISA assays are in line with other studies in which variation between results of different HEV ELISA tests have been observed (Rossi-Tamisier et al., 2013; Krumbholz et al., 2014; Norder et al., 2016; Vollmer et al., 2016b), highlighting the need for developing a gold standard for HEV antibody detection.

6.6 Possibility of zoonotic swine-to-human hepatitis E infections in Finland (I–IV, unpublished)

The study demonstrated that pigs were commonly shedding HEV at the time of transfer to fattening farms, potentially transferring the virus from farrowing farms to fattening farms, and thus, creating a possible risk of infection for pig handlers. Presence of antibodies against HEV has been associated with occupational history of cleaning pig barns and assisting sows at birth in Moldova (Drobeniuc et al., 2001) and with pig farming in Denmark (Christensen et al., 2008). In addition, in Estonia, HEV seroprevalence in pig farm workers was significantly higher than that in hunters (Ivanova et al., 2015).

HEV-infected pigs that arrive at fattening farms may infect pigs arriving there from HEV-unexposed farms. Although only a few HEV RNA-positive finisher pigs were detected in the study, infection at a later age during the fattening stage must be considered possible, which constitutes a risk for HEV entering the food chain in pork and pork-derived products at the time of slaughter. In several other countries this risk has been demonstrated by finding HEV RNA, in addition to feces, also in the livers of slaughtered pigs as well as in pig livers sold in retail shops, with varying prevalences. In the UK, 3% of livers collected at a slaughterhouse (Berto et al., 2012b) and 1.3% of those collected from retail shops were positive (Banks et al., 2010). In Canada, the corresponding prevalences were 21% (Leblanc et al., 2010) and 5.7–8.8% (Wilhelm et al., 2014; 2015), respectively. In France, 0–75% of livers collected at slaughterhouses were positive, depending on the farm from which the pigs originated (Rose et al., 2011; Walachowski et al., 2013; Jori et al., 2016). Of pig livers collected from retail shops, 4% in Germany (Wenzel et al., 2011), 6.5% in the Netherlands (Bouwknegt et al., 2007), and 11.0% in the USA (Feagins et al., 2007) were positive. Moreover, HEV RNA was detected in 44.4% of serum samples from slaughtered pigs in Scotland (Crossan et al., 2015). HEV RNA has also been detected in swab samples from slaughterhouse workers (Berto et al., 2012b) and slaughterhouse floors as well as in surface swab samples from slaughterhouse tools such as knives and belts (Di Bartolo et al., 2012), a metal point used to hook the carcasses at a meat processing plant, and knives and slicers at points of sale (Berto et al., 2012b). Slaughterhouse workers have been shown to be significantly more often HEV antibody-positive than the control group (Krumbholz et al., 2012). The true significance of this risk in Finland requires further studies on HEV occurrence in slaughter-aged pigs and pork-derived foodstuff. In addition, it is still unclear how HEV persists in farm surroundings, and thus, examination of transportation vehicles of HEV and means of prevention of the infection in the swine production chain, starting with assessment of the types of control measures that could be used to reduce HEV occurrence and transmission on farrowing farms, is warranted.

No confirmed zoonotic HEV-3 infections acquired in Finland were detected in humans in this study. However, an acute, symptomatic, and locally acquired human case of HEV-3 infection has been diagnosed in Finland (Kettunen et al., 2013), which demonstrated that the zoonotic HEV-3 common in swine also infects humans in Finland. Finnish veterinarians commonly have antibodies against HEV, and although the results of the analysis of possible risk factors of HEV infection were contradictory, they suggested that contact with blood or tissue fluid from swine might be a risk factor for HEV infection of veterinarians. The fact that antibodies against HEV, and thus, HEV infections are highly common in production pigs in Finland suggests that swine may play a

role in the HEV infections of humans. However, the significance of these observations requires more detailed studies for confirmation. Further studies are also needed to investigate whether veterinary work constitutes a risk factor for HEV infection in Finland, as veterinarians have been demonstrated to have antibodies against HEV more often than control groups in studies conducted in the USA (Meng et al., 2002), the Netherlands (Bouwknegt et al., 2008a), and France (Chaussade et al., 2013).

Diagnosing acute HEV-3 infections in humans is challenging because of their often asymptomatic or mildly symptomatic and self-limiting nature in otherwise healthy persons (Kamar et al., 2012), which is why infected individuals may fail to contact healthcare. Nevertheless, since these infections can have severe outcomes especially in immunocompromised individuals (Kamar et al., 2008; Dalton et al., 2009); acute or subacute liver failure in patients with pre-existing liver disease and chronic infections in organ transplant recipients (Kamar et al., 2012), and can also cause serious illness in otherwise healthy persons (Kettunen et al., 2013), controlling the risk of zoonotic and foodborne HEV infections is important. In Finland, the possibility of HEV infection should be taken into account when diagnosing human patients with hepatitis symptoms, and since HEV is very common in pigs, the possibility of locally acquired and zoonotic human hepatitis E cases should not be overlooked.

7 Conclusions

Hepatitis E virus is present in Finland. HEV-3 subtype e is common in production pigs, and the majority, 85.2%, of slaughter-aged pigs and sows have antibodies against HEV. Finnish veterinarians commonly have antibodies against HEV, with apparent seroprevalence of 10.2%, and markers for hepatitis E infection were present in 27.6% of human patients diagnosed with unexplained acute hepatitis, with markers for acute hepatitis E infection in 11.3% of the patients.

Pigs were infected either at the end of the farrowing period on farrowing farms or at the beginning of the fattening period on fattening farms, at an age of 2–3 months, when the prevalence of HEV RNA-positive pigs was at its peak, 34.6%. Pigs excreted HEV in their feces until approximately 4 months of age, and only 2.9% of HEV RNA-positive slaughter-aged pigs were detected, suggesting that pigs are usually cleared from the infection by this time. There seems to be both HEV-positive and -negative swine farms in Finland, and HEV-3 subtype e isolates from different farms formed genetic clusters of their own. When HEV RNA-negative pigs were transferred to the swine test station, where they came into contact with HEV RNA-positive pigs, pig-to-pig transmission of the infection was observed. This may indicate that infection at a later age during the fattening stage must also be considered possible, constituting a risk for HEV entering the food chain in pork and pork-derived products at the time of slaughter. In addition, common shedding of HEV in the feces of pigs at the time of transfer from farrowing to fattening farms creates a possible risk of zoonotic infection for pig handlers.

Among veterinarians, surprisingly, HEV seropositivity was associated with working as a small animal practitioner and negatively associated with having contacts with swine. However, the seroprevalence appeared to be higher in those who had had needle stick by a needle previously injected into a pig than in those who had not, suggesting that contact with blood or tissue fluid from swine might be a risk factor for HEV infection. No confirmed zoonotic or autochthonous HEV-3 infections in humans were detected. From patients with unknown acute hepatitis, five isolates of HEV-1 were obtained, and the fact that most of the patients with acute infection had recently visited HEV-1 endemic areas in Asia, Africa, or Mexico indicated that the infections were obtained during travels. Traveling outside Europe should be considered when investigating the sources of HEV infections of veterinarians, too, since the seroprevalence appeared to be higher among those small animal practitioners who had traveled outside Europe than among those who had not. Thus, although pigs seem to play a role in the hepatitis E infections of veterinarians, direct contact with pigs is probably not the only HEV exposure, but possibly multiple factors, also including HEV reservoirs other than pigs, are involved.

HEV must be considered a possible cause of acute hepatitis in humans in Finland, especially in patients who have returned from HEV-1 and HEV-2 endemic areas, but the possibility of locally acquired zoonotic hepatitis E infections should not be overlooked. However, the true significance of the risk of zoonotic and foodborne hepatitis E infections in Finland requires further studies on HEV occurrence in slaughter-aged pigs and pig-derived foodstuff, in addition to other animal reservoirs of HEV, especially in wild boars and deer.

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